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<p>(54) Title: <b>CELLULAR FLIP COMPOSITIONS FOR TREATMENT OF ARTERIOSCLEROTIC DISORDERS</b> (57) Abstract <p>A method for treating conditions associated with vascular wall inflammation, particularly arteriosclerosis and vascular injury is provided. The method involves administering to subjects in need of such treatment an effective amount of a FLIP molecule.</p></p>		

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**CELLULAR FLIP COMPOSITIONS**  
**FOR TREATMENT OF ARTERIOSCLEROTIC DISORDERS**

**Related Applications**

5        This application claims priority under 35 USC § 119(e) from U.S. Provisional Patent Application Serial No. 60/075,471 filed on February 20, 1998, entitled CELLULAR FLIP COMPOSITIONS FOR TREATMENT OF ARTERIOSCLEROTIC DISORDERS. The content of the provisional application is hereby expressly incorporated by reference.

**Field of the Invention**

10        This invention relates to methods and compositions for the treatment of arteriosclerotic disorders, such as the excessive death and damage of endothelial cells associated with atherosclerosis. The methods involve administering a cellular FLIP molecule to inhibit apoptosis in vascular endothelial cells.

**Background of the Invention**

15        Arteriosclerosis is a disease that is characterized by a thickening and hardening of regions of an arterial wall. Atherosclerosis is a type of arteriosclerosis that affects the large arteries and is often the basis for coronary artery disease, aortic aneurysm, arterial disease of the lower extremities, and cerebrovascular disease. A particular type of atherosclerosis is transplant arteriosclerosis, which refers to atherosclerosis that occurs in the arteries of transplanted organs,  
20        e.g., transplanted hearts and kidneys. Transplant arteriosclerosis is a major cause of death in patients who receive transplanted organs.

      Atherosclerosis is characterized by the formation of fibrous plaques that contain a large number of smooth muscle cells, macrophages, collagen, extracellular lipid, and necrotic cell debris. The accumulation of material in a fibrous plaque results in narrowing of the blood vessel  
25        lumen which, in turn, restricts arterial blood flow. When the fibrous plaques become sufficiently large to block blood flow completely, the organs that are supplied by the artery undergo ischemia and necrosis. The accumulation of fibrous plaques also weakens the artery, an event which frequently results in rupture of the intima, aneurysm and hemorrhage. Moreover, fragments of the fibrous plaque may detach and form arterial emboli that can precipitate an aortic aneurysm  
30        or arterial disease of the lower extremities.

      To date, the most frequently used methods for treating atherosclerosis include surgical procedures, drug therapies, and combinations of the foregoing. In general, the drug therapies for

treating atherosclerosis are designed to prevent or reduce the accumulation of plaque material. For example, drugs such as diuretics, anti-adrenergic agents, vasodilators, angiotensin-converting enzyme inhibitors, renin inhibitors, HMG-CoA reductase inhibitors, and calcium channel antagonists have been used to treat conditions such as hypertension, hyperlipidemia, and hypercholesterolemia, which contribute to the development of atherosclerosis. In addition, anti-inflammatory agents have been prescribed for inhibiting transplant arteriosclerosis; however, this approach has met with limited success. Surgical methods for treating atherosclerosis include coronary bypass surgery, atherectomy, laser procedures, ultrasonic procedures, and balloon angioplasty. Such methods involve significant risk (e.g., of infection, death) to the patient and, even if successful, fibrous plaque formation frequently occurs at the site of vascular anastomoses, causing reclusion of the surgically-treated vessel. Injury to the blood vessel wall, e.g., resulting from balloon angioplasty, has been shown to disrupt the protective layer of endothelial cells that line the vascular wall, thereby leading to further endothelial cell damage and death.

In view of the foregoing, a need still exists to better understand the molecular processes underlying arteriosclerosis, and to develop improved drug therapies to replace or supplement the existing methods for treating these and other conditions that are mediated by endothelial cell death and vascular wall inflammation. Preferably, such drug therapies would be designed to reduce or prevent plaque formation at its earliest stages.

#### Summary of the Invention

The recruitment of leukocytes at sites of vascular inflammation is a multistep process that involves tethering, rolling, firm adhesion, and the migration of these cells to the subendothelial space. Although much is known about the chemoattractant and adhesion molecules that regulate leukocyte recruitment in response to bacterial infection, relatively little is known about mechanisms that may actively control the transendothelial cell migration. The invention is based, in part, upon Applicant's discoveries relating to the roles played by an endothelial cell intracellular protein in protecting the vascular wall from injury and, thereby, inhibiting leukocyte extravasation and vascular wall inflammation.

Applicant has discovered that functional Fas ligand is expressed on the normal vascular endothelium and that expression of the Fas ligand is down regulated by the inflammatory cytokine,  $\text{TNF}\alpha$ . As described in more detail below, Applicant's further discoveries include: (1) adenovirus-mediated constitutive Fas ligand expression by the endothelium reduces the leukocyte extravasation that is induced by local treatment with  $\text{TNF}\alpha$ ; (2) functional Fas ligand-expressing

endothelial cells are not sensitive to Fas ligand-induced apoptosis; (3) dysfunctional Fas ligand-expressing endothelial cells (e.g., cells exposed to oxidized lipid) are sensitive to Fas ligand-induced apoptosis; and (4) dysfunctional endothelial cells (e.g., cells exposed to oxidized lipid) exhibit a higher death rate and reduced cellular FLICE-inhibitory protein (FLIP) mRNA levels compared to endothelial cells that are not so exposed, i.e., oxidized lipid appears to down-regulate FLIP mRNA transcription.

The foregoing observations are useful in understanding the pathogenesis of atherosclerosis and suggest that, in the absence of an inflammatory response, Fas ligand serves an atheroprotective function in the endothelium through its ability to induce apoptosis in mononuclear cells attempting to invade the vessel wall. In contrast, in the presence of an inflammatory response (e.g., secretion of  $\text{TNF}\alpha$  by activated mononuclear cells within an atheroma), Fas ligand expression in adjacent normal endothelium would be down regulated, thereby promoting more leukocyte extravasation and lesion growth. Taken in its entirety, the data presented herein suggests a novel mechanism by which endothelial cells become dysfunctional and susceptible to Fas ligand-mediated apoptosis, namely, down regulation of FLIP expression by agents that are harmful to the endothelium.

Although not wishing to be bound to a particular theory or mechanism, Applicant believes that deregulated expression of the Fas ligand-Fas receptor signaling pathway is a feature of endothelial cell dysfunction in response to injurious agents (e.g., oxidized lipid, mechanical injury, homocysteine, toxins, viruses) and that abnormal FLIP expression plays an important role in the deregulation of the Fas ligand-Fas receptor signaling pathway. In functional endothelial cells, FLIP expression appears to protect the endothelial cells from Fas ligand-mediated apoptosis. In dysfunctional endothelial cells (e.g., cells exposed to oxidized lipids, see the Examples), FLIP expression is down regulated and the endothelial cells are susceptible to Fas ligand-mediated apoptosis. This proposed nexus between FLIP expression and a susceptibility to Fas ligand-mediated apoptosis is supported by Applicant's further results which demonstrate that the effects of oxidized lipids on Fas ligand-induced apoptosis and FLIP expression can be abrogated by first contacting the endothelial cells with a "neutralizing Fas ligand antibody" (an antibody that binds to the Fas ligand and prevents binding of the ligand to the Fas receptor).

FLIP is an anti-apoptotic protein that recently has been reported in the literature (Irmeler, M., et al., *Nature* 388:190-195 (1997); Shu, H., et al., *Immunity* 6:751-763 (1997) (FLIP is referred to as "Casper" in the Shu reference). To understand how FLIP protects endothelial cells

from apoptosis, it is first necessary to briefly describe the series of events that lead to apoptosis.

In general, the literature reports that the binding of TNF $\alpha$  (tumor necrosis factor) to TNF $\alpha$  receptors expressed on a target cell surface results in triggering an intracellular signaling pathway that leads to the activation of enzymes (e.g., caspases) that are central to the process of programmed cell death. The intracellular protein interactions that are triggered by, e.g., TNF binding to its receptor reportedly can be attributed to two structural motifs within the proteins concerned (D. Wallach, *Nature* 388:123-124 (1997)). The first motif is referred to as the "death domain" (DD) and is found in several death-inducing receptors of the TNF family, including the Fas receptor (also referred to as CD95 or Apo-1), as well as in several cytoplasmic adaptor proteins that bind through this domain both to receptors and to each other (e.g., MORT-1/FADD and TRADD adaptors). The second motif is referred to as the "death effector domain" (DED). A "death effector domain" is found in MORT-1/FADD upstream of the death domain and occurs in duplicate in two caspases: caspase 8 (MACH/FLICE/Mch-5) and caspase 10 (Mch-4/FLICE-2). Reportedly, the binding of these caspases to MORT-1/FADD through association of their "death effector domain" motifs and consequent activations of these caspases (reportedly by autoproteolytic cleavage), are critical steps in the initiation of apoptosis.

FLIP contains "death effector domain" (DED) motifs at its amino terminus, through which FLIP can bind to other DED-containing proteins. Despite this sequence similarity to known proteins, there exists no consensus on the physiological role of FLIP. Irmeler et al. propose that FLIP blocks death induction by interfering with the binding of DED-containing caspases to MORT-1/FADD and that binding of the carboxy-terminal region in FLIP to caspase 8 and caspase 10 may constitute a further inhibitory mechanism (by preventing the proteolytic self-processing of these enzymes) (Irmeler et al., *Nature* 388:190-195 (1997)). In contrast, Shu et al., propose that FLIP acts solely as a death activator (Shu, H., et al., *Immunity* 6:751-763 (1997)). Various other groups have described the FLIP protein and have attributed to it different properties, as well as different names ("CASH", "FLAME", "I-FLICE"; as noted in D. Wallach, *Nature* 388:123-124 (1997)). The Sequence Listing contains the GenBank sequences for several FLIP isoforms that have been reported in the literature.

Although not intending to be bound to a particular theory or mechanism, Applicant believes that oxidized lipid mediates endothelial cell death by inhibiting (directly or indirectly) FLIP transcription, thereby *rendering the endothelial cell susceptible to Fas ligand-induced apoptosis*. Thus, Applicant describes herein a newly discovered function for cellular FLIP,

namely, the ability to protect vascular endothelial cells from Fas ligand-induced apoptosis and, thereby, maintain the integrity of the vascular wall. Accordingly, the invention is directed to delivering FLIP molecules (nucleic acids, polypeptides) to endothelial cells to inhibit apoptosis that is mediated by the Fas ligand-Fas receptor signalling pathway. The instant invention  
5 embraces compositions and methods that are based upon the discovery of this newly-discovered function for cellular FLIP.

According to one aspect of the invention, a method for treating a subject diagnosed as having a condition characterized by vascular wall inflammation and, optionally, elevated vascular levels of oxidized lipid, is provided. Exemplary conditions that are characterized by vascular wall  
10 inflammation include: atherosclerosis, transplant arteriosclerosis (e.g., heart transplant, kidney transplant), and conditions associated with vascular injury resulting from, for example, balloon angioplasty. The method involves administering to the subject an isolated FLIP molecule (a "FLIP nucleic acid" or a "FLIP polypeptide") in an amount effective to inhibit Fas ligand-mediated apoptosis of vascular endothelial cells in vivo. Preferably, the FLIP molecule is  
15 administered to the subject in conjunction with a method for treating an arteriosclerotic condition. The method for treating an arteriosclerotic condition may be a surgical method or a drug therapy (e.g., gene therapy). The compositions and methods of the invention are useful for replacing existing drug therapies, as well as for improving the effectiveness of existing therapies for treating conditions that are characterized by excessive vascular smooth muscle cell proliferation. In  
20 general, such conditions are diagnosed by detecting the presence of fibrous plaques in the blood vessel walls of the subject.

In the particularly preferred embodiments, the FLIP molecule is delivered directly to the site at which there is vascular wall inflammation or a predisposition to vascular wall inflammation, e.g., the arteries of a transplanted organ, the site of a vascular injury. For example,  
25 prophylactic treatment for transplant arteriosclerosis can be accomplished by perfusing a transplant organ with a FLIP molecule (preferably a FLIP nucleic acid contained in an expression vector) prior to transplanting the organ into the patient. Alternatively, delivery of a FLIP molecule into the arteries of a subject can be accomplished by attaching a FLIP nucleic acid or a FLIP polypeptide to the surface of a balloon catheter, inserting the catheter into the subject until  
30 the balloon portion is located at the site of an occlusion (or a predisposition to an occlusion), and inflating the balloon to contact the balloon surface with the vessel wall at the site of the occlusion. In this manner, the compositions can be targeted to particular sites within a vessel to prevent or

reduce leukocyte infiltration and smooth muscle cell proliferation at these sites. Optionally, the FLIP molecule is delivered in combination with a cytokine that promotes endothelial cell proliferation, or a nucleic acid encoding a cytokine that promotes endothelial cell proliferation.

A "FLIP molecule" embraces a "FLIP nucleic acid" and a "FLIP polypeptide". As used  
5 herein, a "FLIP nucleic acid" refers to a nucleic acid molecule which: (1) has the sequence of SEQ. ID NO. 1 (the "Homo sapiens FLICE-like inhibitory protein long form mRNA, complete CDs" having GenBank Accession No. U97074 human FLIP molecule) or hybridizes under stringent conditions to a cDNA that is transcribed into SEQ. ID NO. 1 and (2) codes for a FLIP  
10 polypeptide that inhibits (prevents or reduces) Fas ligand-mediated apoptosis. Preferably, the FLIP nucleic acid inhibits Fas ligand-mediated apoptosis of vascular endothelial cells. More preferably, the FLIP nucleic acid encodes one or more death effector domains, alone or in combination with one or more caspase-like subunits. The preferred FLIP nucleic acid has the sequence of SEQ. ID NO.1 (FLIP-long form). Homologs and alleles of a nucleic acid having the sequence of SEQ. ID NO.1 also are embraced within the definition of a "FLIP nucleic acid".  
15 In addition, the FLIP nucleic acids of the invention include nucleic acids which code for the FLIP polypeptide having the sequence of SEQ. ID NO.2 (the polypeptide sequence encoded by SEQ. ID NO.1), but which differ from the sequence of SEQ. ID NO.1 in codon sequence due to the degeneracy of the genetic code. The invention also embraces isolated functionally equivalent fragments, variants, and analogs of the foregoing nucleic acids; proteins and peptides coded for  
20 by any of the foregoing nucleic acids; and complements of the foregoing nucleic acids. Particularly preferred fragments of the FLIP nucleic acid and FLIP polypeptides that are embraced by the invention are discussed below.

At least five different FLIP isoforms have been reported in the literature:

(1) I-FLICE Isoform 1 (alternatively referred to as "FLIP-L", "FLIP-long form", "MRIT- $\alpha$ 1",  
25 "CASPER", "FLAME-1 $\gamma$ " and CASH- $\alpha$ ); (2) FLIP-S (alternatively referred to as "FLIP-short form", CASH- $\beta$ ); (3) FLAME-1 $\alpha$ ; (4) FLAME-1 $\beta$ ; and (5) FLAME-1 $\delta$ . The nucleic acid and/or amino acid sequences for these isoforms have been reported in the literature or have been deposited at GenBank. In the preferred embodiments, the FLIP molecules are based upon the sequences of the FLIP-long form or the FLIP-short form isoforms. GenBank Sequences for these  
30 and other representative isoforms are provided in the Sequence Listing.

The FLIP isoforms have in common an exon encoding a sequence containing 202 amino acids which includes two "death effector domains" (DED). As used herein, a "FLIP polypeptide"



refers to a polypeptide that is coded for by a FLIP nucleic acid and that includes one or more DED motifs. Exemplary DED sequences are provided in SEQ. ID NO. 1 where "DED I" corresponds to amino acids 1-74 and "DED II corresponds to amino acids 93-171; the numbering is based upon that reported by M. Irmeler, et al., in *Nature* 388:190-195 (1997) for the human cellular FLIP.

Alternative sequences that are homologous to those provided in SEQ. ID NO. 1 and that have the same functional activity of the DED I and DED II also are embraced within the meaning of the phrase, "death effector domain", as used herein. The exact amino acid positions which define the limits of the DED sequences in various FLIP isoforms can be identified by reference to the sequences for these proteins (see, e.g., GenBank or the Sequence Listing) and using routine procedures to identify those sequences which have sequence homology to the DED sequences of SEQ. ID NO. 1.

The preferred FLIP polypeptides further include one or more carboxy-terminal regions present in, e.g., SEQ. ID NO. 1, and referred to herein as "caspase-like domains", that resemble the carboxy-terminal protease-precursor regions in caspase 8 and caspase 10 but that lack several of the sequence features required for protease activity. For example, the FLIP caspase-like domain contains a Tyr residue (amino acid position 360 in SEQ. ID NO. 2, human FLIP-L), corresponding to the active-site cysteine of FLICE, and an Arg (amino acid position 315 in SEQ. ID NO. 1, human FLIP) or a Leu (amino acid position 317 in SEQ. ID NO. 6, murine FLIP) corresponding to an active-site histidine of FLICE. The caspase-like domains further contain subunits ("caspase-like subunits") which, in the enzymatically active caspase, represent the proteolytic cleavage fragments of the enzyme.

FLIP polypeptides are useful for inhibiting Fas-ligand mediated apoptosis and, in particular, are useful for inhibiting apoptosis in vascular endothelial cells. The preferred FLIP polypeptides of the invention have the amino acid sequence of SEQ. ID NO.2 (the sequence for the human FLIP-L polypeptide) or SEQ. ID NO. 4 (the sequence for the human FLIP-S polypeptide). The sequences for the murine FLIP nucleic acid and corresponding polypeptide are provided in GenBank database and are included in the SEQUENCE LISTING. FLIP polypeptides further embrace functionally equivalent fragments, variants, and analogs of SEQ. ID NOs. 2 and 4, provided that the fragments, variants, and analogs inhibit Fas ligand-mediated apoptosis and include one or more "death effector domain" motifs. The invention also embraces proteins and peptides coded for by any of the foregoing nucleic acids. For example, the invention

embraces proteins and polypeptides which are coded for by unique fragments of the foregoing nucleic acids. Such proteins and polypeptides are useful, for example, as immunogens for generating antibodies to unique epitopes of the FLIP polypeptide.

According to one embodiment of the invention, a method for inhibiting (preventing or  
5 reducing) Fas ligand-mediated apoptosis of vascular endothelial cells in vivo or in vitro is provided. The method involves contacting an endothelial cell with an isolated FLIP nucleic acid or polypeptide under conditions to permit the introduction into the endothelial cell of a functional FLIP nucleic acid or FLIP polypeptide. For example, a FLIP nucleic acid contained in an  
10 expression vector can be introduced into the cell, followed by allowing the cell to transcribe and translate the FLIP nucleic acid into a FLIP polypeptide in the endothelial cell. Alternatively, a FLIP polypeptide contained in a liposome can be introduced into the cell. The FLIP molecule is introduced into the cell in an amount sufficient to inhibit Fas ligand-mediated apoptosis of the endothelial cell in vivo or in vitro. In the preferred embodiments, the FLIP molecule is introduced into the cell in an amount sufficient to inhibit apoptosis that is mediated by contacting  
15 the cell with an oxidized lipid (e.g., oxidized LDL).

According to certain other embodiments of the invention, an isolated FLIP nucleic acid is administered to a subject in need thereof in an amount effective to inhibit (prevent or reduce) Fas ligand-mediated apoptosis of vascular endothelial cells in vivo. The subjects are treated with the FLIP nucleic acid in a manner and in an amount so as to inhibit vascular endothelial cell  
20 apoptosis, preferably at a site of vascular wall inflammation or a predisposition to vascular wall inflammation, while minimizing the potential for systemic toxicity. Further specificity of treatment is achieved by operably coupling the FLIP nucleic acid to an inducible promoter or a tissue-specific promoter, such as an endothelial cell-specific promoter. Preferably, the promoter is one which is not down regulated by oxidized lipid (e.g., strong viral promoters such as CMV).

According to yet other embodiments of the invention, an isolated FLIP polypeptide is  
25 administered to a subject in need thereof in an amount effective to inhibit Fas ligand-mediated apoptosis of vascular endothelial cells in vivo. The subjects are treated with the FLIP polypeptide in a manner and in an amount so as to inhibit vascular endothelial cell apoptosis, preferably at the site of vascular wall inflammation or a predisposition to vascular wall inflammation, while  
30 minimizing the potential for systemic toxicity. The FLIP polypeptides are delivered in a vehicle which permits cytoplasmic delivery of the FLIP polypeptide. Exemplary vehicles that can be used for this purpose include liposomes. Liposomes are commercially available from Gibco

BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleoyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications. Liposomes also have been reviewed by Gregoriadis, G. in *Trends in Biotechnology*, V. 3, p. 235-241 (1985).

The complete coding sequence for a human FLIP mRNA and predicted amino acid sequence have been assigned GenBank Accession No. U97074 and are provided herewith as SEQ. ID NOS. 1 and 2 (human FLIP-L nucleic acid and polypeptide), respectively. The preferred FLIP nucleic acids of the invention encode the FLIP polypeptides having the amino acid sequence of SEQ. ID NO. 2 (human FLIP-L), homologs and alleles of SEQ. ID NO. 2, or functionally equivalent fragments or variants of SEQ. ID NO. 2. Also preferred are human FLIP-short form ("FLIP-S") nucleic acids and polypeptides (SEQ. ID Nos. 3 and 4, respectively), homologs, alleles, and functionally equivalent fragments and variants of the foregoing. Various other embodiments of the invention are directed to compositions and/or methods use these and other mammalian FLIP isoforms as provided in the GenBank database (see also Sequence Listing), as well as viral FLIP molecules (e.g., GenBank accession no. U20824).

Preferably, the FLIP nucleic acid has the nucleotide sequence of SEQ. ID NO 1 (human FLIP-L), i.e., the complete coding sequence of the mRNA encoding the "intact human FLIP-L". The intact human FLIP-L polypeptide (SEQ. ID NO. 1) contains two "death effector domains" (DED I and DED II) and a caspase-like domain containing two caspase-like subunits ("p17 subunit", "p12 subunit") that have homology to the p17 and the p12 subunits, respectively, of FLICE. The amino acid sequences of these domains as defined in reference to SEQ. ID NO. 2 are as follows: DED I contains amino acids 1 to 74; DED II contains amino acids 93 to 171; homology to the p17 FLICE subunit is from amino acid 242 to 376 in FLIP-L; homology to the p12 FLICE subunit is from amino acid 377 to 480 in FLIP-L; the numbering is based upon that reported by M. Irmeler, et al., in *Nature* 388:190-195 (1997) for the human cellular FLIP. Throughout this application, the sequence numbers that are provided are intended to be inclusive, e.g., DED I contains amino acids 1 to 74, inclusive.

The isolated nucleic acids of the invention also include nucleic acids encoding fragments of an intact FLIP. For example, the FLIP nucleic acid may encode one or more DED domains, alone or in combination with one or more caspase-like subunits. Thus, exemplary FLIP nucleic acids include those which encode: DED I, DED II, DED I coupled to DED II, multiples of the

foregoing DED domains, alone or in combination with one or more of the caspase-like subunits p17 and p12. Preferably, the FLIP nucleic acids encode DED I (amino acids 1-74), DED II (amino acids 93-171), and DED I coupled to DED II (e.g., amino acids 1-74 coupled directly to amino acids 93-171 of SEQ. ID NO. 1, amino acids 1-171 of SEQ. ID NO. 1). No prior use for  
5 the such isolated fragments of cellular FLIP nucleic acids or polypeptides disclosed herein has been proposed. Optionally, the FLIP nucleic acids further contain one or more caspase-like subunits.

FLIP polypeptide fragments that are "FRAGMENT 1" contain DED I and DED II (e.g., amino acids 1 to 171, with or without intervening amino acids 75-92 of FLIP-L, SEQ. ID NO.2)  
10 but do not include one or more of caspase-like domains (e.g., amino acids 172 to the carboxyl-terminus of SEQ. ID NO.2, as numbered by Irmeler et al., *Nature, supra.*). FLIP FRAGMENT 1 polypeptides previously have not been described. Accordingly, one particular aspect of the invention relates to such FLIP FRAGMENT 1 polypeptides, nucleic acids encoding same, complements of said nucleic acids, vectors containing said nucleic acids, host cells containing  
15 said vectors, antibodies that selectively bind to said polypeptides but that do not bind to the intact FLIP polypeptides, and methods for using the foregoing compositions. The invention further embraces nucleic acid molecules that differ from the foregoing isolated nucleic acid molecules in codon sequence due to the degeneracy of the genetic code. Throughout this document, it is intended that the cDNAs corresponding to the mRNAs that are provided in the sequences also are  
20 embraced within the meaning of the phrase, "FLIP nucleic acid".

In the preferred embodiments of the methods, the FLIP nucleic acid is selected from the group consisting of an intact FLIP-L nucleic acid (e.g., SEQ. ID NO.1, the coding region of SEQ. ID NO.1), a FLIP FRAGMENT 1 containing DED I and DED II (e.g., the nucleic acid sequence of SEQ. ID NO. 1 that encodes amino acids 1 to 171 of SEQ. ID NO. 2), a FLIP FRAGMENT  
25 2 containing DED I but excluding DED II (e.g., the nucleic acid sequence of SEQ. ID NO. 1 that encodes amino acids 1 to 74 of SEQ. ID NO. 2), a FLIP FRAGMENT 3 containing DED II but excluding DED I (e.g., the nucleic acid sequence of SEQ. ID NO. 1 that encodes amino acids 93 to 171 of SEQ. ID NO. 2), as well as the foregoing FLIP FRAGMENTS further including one or more caspase-like subunits (e.g., the nucleic acid sequence of SEQ. ID NO. 1 that encodes  
30 amino acids 242 to 376 and/or amino acids 377 to 480 of SEQ. ID NO. 2).

The preferred FLIP nucleic acid fragments encode a functionally equivalent fragment of an intact FLIP. The FLIP nucleic acid is operatively coupled to a promoter that can express the

FLIP in a targeted cell (e.g., a vascular endothelial cell). Preferably, the nucleic acid is contained in an appropriate expression vector (e.g., adenoviral vector, modified adenoviral vector, retroviral vector, plasmid, liposome) to more efficiently genetically modify the targeted cell and achieve expression of the FLIP on the targeted cell surface. More preferably, the FLIP nucleic acid promoter is one which is not down regulated, directly or indirectly, by oxidized lipid, e.g., the CMV promoter.

Applicant has discovered that vascular endothelial cells express the Fas receptor and that such cells become susceptible to Fas ligand-mediated apoptosis under conditions of reduced FLIP expression (e.g., conditions associated with elevated vascular oxidized lipid levels). As a result, delivery of a nucleic acid encoding the FLIP polypeptide to a site of vascular wall inflammation (or a predisposition to vascular wall inflammation) serves to inhibit Fas ligand-mediated apoptosis of vascular endothelial cells, thereby promoting the beneficial effects of viable endothelial cells in the vessel wall. Thus, according to one aspect of the invention, a method for treating vascular wall inflammation in a subject is provided. A FLIP nucleic acid is administered to a subject in need of such treatment in an amount effective to inhibit Fas ligand-mediated apoptosis of vascular endothelial cells. The preferred FLIP nucleic acids are as described herein. This method is useful, for example, for treating atherosclerosis and transplant arteriosclerosis.

According to a related aspect of the invention, a method is provided for treating a subject that has sustained a vascular injury which results in, or is otherwise associated with, vascular wall inflammation. A FLIP nucleic acid is administered to a subject in need of such treatment in an amount effective to inhibit Fas ligand-mediated apoptosis of vascular endothelial cells resulting from, or associated with, the injury. The FLIP nucleic acid preferably is as described herein. In one embodiment, the FLIP nucleic acid is administered to a subject with an arterial occlusion in conjunction with treatment of that occlusion. The occlusion can be a coronary artery occlusion and the treatment can be dilation balloon angioplasty.

It is to be understood that a FLIP polypeptide can be used in place of a FLIP nucleic acid in treating any of the foregoing conditions. Thus, according to still another aspect of the invention, pharmaceutical preparations are provided that contain a FLIP nucleic acid or a FLIP polypeptide. Preferably, the FLIP polypeptides are contained in a vehicle (e.g., liposome) that is capable of delivering the FLIP polypeptide into the cytoplasm of the endothelial cell. The pharmaceutical preparations contain the above-described FLIP molecules, together with a pharmaceutically-acceptable carrier. Preferably, the FLIP molecules are present in the

compositions in an amount effective for treating a condition that is characterized by vascular wall inflammation. The FLIP molecules are particularly useful for the treatment of atherosclerosis and transplant arteriosclerosis. The effective amount is sufficient to inhibit Fas ligand-mediated apoptosis of vascular endothelial cells in vivo.

5 According to still another aspect of the invention, the above FLIP molecules (FLIP nucleic acids and FLIP polypeptides) are used in the preparation of medicaments, preferably for the treatment of conditions characterized by vascular wall inflammation. Such conditions include atherosclerosis and transplant arteriosclerosis. Atherosclerosis is associated with the following conditions, each of which can be treated using the compositions of the invention: restenosis,  
10 pulmonary hypertension, and vascular remodeling. The method involves placing the FLIP molecules in a pharmaceutically-acceptable carrier. The preferred FLIP molecules are as described herein.

It is noted that the preferred subjects treated according to the methods set forth above are otherwise free of symptoms calling for FLIP treatment, either by administration of the FLIP  
15 polypeptide or by a FLIP nucleic acid.

The invention also contemplates the use of FLIP molecules in experimental model systems to determine the role that vascular endothelial cells play in the repair of an injury to a vessel wall or in mediating an adverse health consequence occurring as a result of organ transplant. An injury to a blood vessel of an animal or a pulmonary hypertensive state is induced  
20 experimentally, for example, by hypercholesterolemic diet and/or by inducing a hypoxic state at a particular site. A FLIP molecule as described above then is administered to the animal. The application may be local or may be systemic. Then the animal's response is monitored and compared to control animals that do not receive the FLIP molecules. Similarly, organ transplant can be performed in an animal model, with or without treatment of the organ and/or animal with  
25 a FLIP molecule. The application of the FLIP molecule may be local or may be systemic. Then the animal's response is monitored and compared to control animals (and/or control organs) that do not receive the FLIP molecules to assess the effectiveness of FLIP in inhibiting Fas ligand-mediated apoptosis of vascular endothelial cells in vivo.

According to still another aspect of the invention, a screening method for selecting an  
30 inhibitory agent that inhibits the development or progression of atherosclerotic lesions is provided. The method involves: (1) contacting a cell that expresses FLIP with an oxidized lipid and a putative inhibitor under conditions wherein the cell is capable of undergoing Fas ligand-

induced apoptosis; (2) determining whether the cell undergoes Fas ligand-induced apoptosis in the presence of the putative inhibitor; and (3) selecting the putative inhibitor that inhibits or prevents Fas ligand-induced apoptosis as an agent that inhibits the development or progression or atherosclerotic lesions. In a particularly preferred embodiment, the putative inhibitor is a relatively small synthetic compound and, more preferably, is contained in a combinatorial or peptide library that contains a mixture of at least ten different compounds.

According to yet another aspect of the invention, a kit is provided. The kit includes: (1) an oxidized lipid (e.g., oxidized LDL); (2) a cell that expresses FLIP and that is capable of undergoing Fas ligand-induced apoptosis when FLIP expression is down regulated (e.g., an endothelial cell); (3) a Fas ligand or a Fas ligand mimic (e.g., antibody that binds to Fas receptor and induces apoptosis); and (4) instructions for inducing apoptosis and determining whether a test compound inhibits or prevents Fas ligand-induced apoptosis.

These and other aspects of the invention will be described in greater detail below. Throughout this disclosure, all technical and scientific terms have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains unless defined otherwise.

All documents identified in this application are incorporated in their entirety herein by reference.

#### **Brief Description of the Drawings**

Figure 1 is a series of graphs illustrating that OxLDL and LPC upregulate FasL expression on human endothelial cells. HUVECs were analyzed with a fluorescence-activated cell sorter (FACS) using antibody against human FasL combined with FITC. Basal FasL expression was detected on untreated cells (Fig. 1A), and cell surface expression was increased in cultures treated with OxLDL (Fig. 1B) or LPC (Fig. 1C).

Figure 2 is a series of graphs illustrating that OxLDL induces apoptosis in human endothelial cells through activation of the Fas/FasL pathway.

Figure 2A is a series of graphs illustrating that OxLDL induces DNA fragmentation. HUVECs (70% confluent) were incubated in the presence or absence of OxLDL (300 µg protein/ml), a neutralizing anti-FasL antibody (10 µg/ml, 4H9) or an agonistic anti-Fas antibody (0.5 µg/ml, CH11, MBL) for 16 hours in combinations as indicated. Hamster IgG was used as an isotype-matched control for 4H9. Floating and attached cells were harvested by trypsinization, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, 0.1% sodium citrates, and

incubated with TdT-mediated dUTP nick end labeling (TUNEL) solution (Boehringer Mannheim) in the absence (open curves) and in the presence (semi-filled curves) of terminal deoxynucleotidyl transferase. Fluorescence intensity was analyzed by FACS.

Figure 2B is a bar graph illustrating that OxLDL impairs cell viability. HAECs were  
5 cultured in a 96-well plate at 80% confluency and treated with OxLDL (300  $\mu$ g protein/ml), a neutralizing anti-FasL antibody (10  $\mu$ g/ml, 4H9) and an anti-Fas antibody (0.5  $\mu$ g/ml, CH11) for 18 hours in combinations as indicated. Cell viability was determined by means of MTT (Dimethylthiazol-diphenyltetrazolium bromide) assay (Tanaka *et al.*, 1996). Data are presented as mean  $\pm$  S.E.M.

10 Figure 3 is a series of graphs illustrating the dose-response and time course relationships for lipid-induced reduction in HUVEC viability. MTT assays were performed to determine cell viability. Dose-response curves were performed on HUVECs after 12 hours incubation with OxLDL (Fig. 3A) or LPC (Fig. 3B, 3C). Time course measurements were made with 70  $\mu$ M LPC.

15 Figure 4 is a schematic representation illustrating the effects of LPC on the expression of various death ligand/death receptor pathway components. Ribonuclease protection assays were performed to determine the effects of LPC on the indicated mRNA transcripts. HUVECS were treated with LPC (70  $\mu$ M) for the indicated times prior to RNA extraction. LPC had little or no effect on the expression of many of the cell death pathway components.

20 Figure 5 is a schematic representation illustrating that LPC downregulates FLIP and upregulates FasL transcript expression. HUVECS were treated with LPC (70  $\mu$ M) for the indicated times prior to RNA extraction. Northern blot analysis was performed using FLIP or FasL cDNA probes. Two FLIP transcripts were detected: FLIP<sub>L</sub> (upper) and FLIP<sub>S</sub> (lower). The 28S ribosome band is shown to indicate equal loading.

25 Figure 6 is a graph illustrating the results of an established cell death assay involving plasmid expression vectors (1-3) to test whether FLIP expression promotes cell viability in the presence of LPC. This cell death assay involves the co-transfection of the test plasmid containing FLIP (long or short), or the apoptosis inhibitor Bcl-X<sub>L</sub>, downstream from the CMV promoter in pCDNA along with a plasmid that expresses  $\beta$ -galactosidase ( $\beta$ -gal), which marks the transfected  
30 cell. Plasmids were co-transfected into cultures of HUVECs. After 24 hours, cells were incubated in the presence of 70  $\mu$ M LPC. 48 hrs post-transfection, cells were fixed in buffered formalin and stained with X-gal. Quantitative analysis of the  $\beta$ -gal-positive cells is an indication



of viability. These assays revealed greater  $\beta$ -gal-positive cells in cultures co-transfected with FLIP or Bcl-X<sub>L</sub> expression vectors than with the control vector (pCDNA) or mock transfected (TE). This increase in the number of surviving  $\beta$ -gal-positive cells indicates that plasmid-mediated FLIP expression can reverse the apoptosis caused by LPC, a component of OxLDL.

Figure 7 schematically illustrates a representative Adeno-FLIP construct according to the invention.

#### Brief Description of the Sequences

SEQ ID NO:1 is the nucleic acid encoding human FLIP-L and having GenBank Accession No. U97074.

SEQ ID NO:2 is the human FLIP-L polypeptide encoded by SEQ. ID NO. 1 and having GenBank Accession No. U97074.

SEQ ID NO:3 is the nucleic acid encoding human FLIP-S and having GenBank Accession No. U97075.

SEQ ID NO:4 is the human FLIP-S polypeptide encoded by SEQ. ID NO. 3 and having GenBank Accession No. U97075.

SEQ ID NO:5 is the nucleic acid encoding murine FLIP-L and having GenBank Accession No. U97076.

SEQ ID NO:6 is the murine FLIP-L polypeptide encoded by SEQ. ID NO. 5 and having GenBank Accession No. U97076.

SEQ ID NO:7 is the nucleic acid encoding an alternative spliced form of human FLAME-1-delta and having GenBank Accession No. AF009619.

SEQ ID NO:8 is the alternative spliced form of human FLAME-1-delta polypeptide encoded by SEQ. ID NO. 7 and having GenBank Accession No. AF009619.

SEQ ID NO:9 is the nucleic acid encoding human FLAME-1 and having GenBank Accession No. AF009616.

SEQ ID NO:10 is the human FLAME-1 polypeptide encoded by SEQ. ID NO. 9 and having GenBank Accession No. AF009616.

SEQ ID NO:11 is the nucleic acid encoding an alternative spliced form of human FLAME-1-beta and having GenBank Accession No. AF009617.

SEQ ID NO:12 is the alternative spliced form of human FLAME-1-beta polypeptide encoded by SEQ. ID NO. 11 and having GenBank Accession No. AF009617.

of viability. These assays revealed greater  $\beta$ -gal-positive cells in cultures co-transfected with FLIP or Bcl-X<sub>L</sub> expression vectors than with the control vector (pCDNA) or mock transfected (TE). This increase in the number of surviving  $\beta$ -gal-positive cells indicates that plasmid-medicated FLIP expression can reverse the apoptosis caused by LPC, a component of OxLDL.

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SEQ ID NO:12 is the alternative spliced form of human FLAME-1-beta polypeptide encoded by SEQ. ID NO. 11 and having GenBank Accession No. AF009617.

### Detailed Description of the Invention

The invention is based on the discovery that vascular endothelial cells express the Fas receptor but that cellular FLIP protects the endothelial cells from Fas ligand-mediated apoptosis.

Accordingly, the compositions of the invention contain FLIP molecules (FLIP ligand nucleic acids and FLIP ligand polypeptides) and methods for delivering these molecules in vivo or in  
5 Figure 1. OxLDL and LPC upregulate FasL expression on human endothelial cells.

HUVECs were analyzed with a fluorescence-activated cell sorter (FACS) using antibody against human FasL combined with FITC. Basal FasL expression was detected on untreated cells (top panel), and cell surface expression was increased in cultures treated with OxLDL (middle  
10 panel) or LPC (bottom panel).

in vitro for the purpose of inhibiting the Fas ligand-mediated apoptosis of vascular endothelial cells.

Cellular FLIP has only recently been described in the literature and various functions for this newly-discovered protein have been proposed. Applicant has discovered that endothelial cells normally express the Fas receptor but are immune to Fas ligand-mediated apoptosis. Based  
15 upon these observations and the experimental results described herein, it is believed that FLIP's physiological function in endothelial cells is to protect these cells from Fas ligand-mediated apoptosis. Applicant has further discovered that under conditions of elevated oxidized lipid, endothelial cells become susceptible to Fas ligand-mediated apoptosis and that such susceptible cells exhibit reduced FLIP transcription. Although not wishing to be bound to one particular  
20 theory or mechanism, Applicant believes that these observations form the foundation for novel therapeutic methods and related compositions for treating conditions that are characterized by vascular endothelial cell death and vascular wall inflammation.

In one aspect, the invention involves the use of a nucleic acid encoding FLIP ("FLIP nucleic acid") to express one or more copies of the FLIP polypeptide in endothelial cells and, in  
25 particular, to express the FLIP polypeptide in endothelial cells that are susceptible to Fas ligand-mediated apoptosis. Although not intending to be bound to any particular theory or mechanism, it further is believed that infection of the vessel wall with, for example, a FLIP nucleic acid-containing vector (e.g., viral vector, plasmid), results in expression of FLIP polypeptide in the endothelial cells; thereby rendering the cells resistant to the Fas ligand-mediated apoptosis.

30 The human and murine FLIP cDNAs have been isolated and sequenced (Irmeler, M., et al., *Nature* 388:190-195 (1997); Shu, H., et al., *Immunity* 6:751-763 (1997). See also, GenBank Accession No. U97074 (SEQ. ID NOS. 1 and 2) for the human cellular FLIP-L mRNA and

predicted amino acid sequences, respectively; GenBank Accession No. U97075 (SEQ. ID NOS. 3 and 4) for the human cellular FLIP-S mRNA and predicted amino acid sequences, respectively; and GenBank Accession No. U97076 (SEQ. ID NOS. 5 and 6) for the murine cellular FLIP-L mRNA and predicted amino acid sequences, respectively.

5 In one aspect, the invention is directed to a method for treating a subject diagnosed as having a condition characterized by vascular wall inflammation and, optionally, further characterized by elevated vascular levels of oxidized lipids. Exemplary conditions that are caused by vascular wall inflammation are known to those of ordinary skill in the art and include, but are not limited to, the following diseases: arteriosclerosis, including atherosclerosis, transplant  
10 arteriosclerosis; post interventional restenosis or other vessel wall injury-induced excessive vascular smooth muscle cell proliferation resulting from endothelial cell dysfunction. The method involves administering to the subject an isolated FLIP molecule in an amount and in a manner effective to inhibit (prevent or reduce the progression of) Fas ligand-mediated apoptosis of vascular endothelial cells in vivo. For the treatment of transplant arteriosclerosis, the FLIP  
15 molecules may, alternatively, be administered by perfusing or soaking the organ in a solution containing the FLIP molecules prior to implantation.

The FLIP molecules of the invention are administered in effective amounts. An effective amount is a dosage of the FLIP nucleic acid sufficient to provide a medically desirable result. The effective amount will vary with the particular condition being treated, the age and physical  
20 condition of the subject being treated, the severity of the condition, the duration of the treatment, the nature of the concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. For example, in connection with atherosclerosis or restenosis, an effective amount is that amount which slows or inhibits the vascular inflammation that is associated with atherosclerosis or restenosis. Likewise, an effective  
25 amount for treating vascular remodeling would be an amount sufficient to lessen or inhibit altogether endothelial cell apoptosis so as to slow or halt the development of or the progression of vascular remodeling. Thus, it will be understood that the FLIP molecules of the invention can be used to treat the above-noted conditions prophylactically in subjects at risk of developing the foregoing conditions. As used in the claims, "inhibit" embraces all of the foregoing. It is  
30 preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment.

One aspect of the invention involves the use of the FLIP molecules of the invention for

treating subjects who have sustained a vascular injury such as an injury to a blood vessel. Injury to the vascular system can lead to a number of undesirable health conditions, including, for example, forms of atherosclerosis and arteriosclerosis that are associated with vascular wall inflammation. A common injury to the vascular system occurs as a side effect of a medical procedure for treating ischemic heart disease. Ischemia refers to a lack of oxygen due to inadequate perfusion of blood. Ischemia heart disease is characterized by a disturbance in cardiac function due to an inadequate supply of oxygen to the heart. The most common form of this disease involves a reduction in the lumen of coronary arteries, which limits coronary blood-flow.

When ischemic heart disease becomes very serious, management of the disease becomes invasive. Until recently, ischemic heart disease was treated by coronary-artery, bypass surgery. Less invasive procedures, however, now have been developed. These procedures involve the use of catheters introduced into the narrowed region of the blood vessel ("the stenosis") for mechanically disrupting, laser ablating or dilating the stenosis.

The most widely used method to achieve revascularization of a coronary artery is percutaneous transluminal coronary angioplasty. A flexible guide wire is advanced into a coronary artery and positioned across the stenosis. A balloon catheter then is advanced over the guide wire until the balloon is positioned across the stenosis. The balloon then is repeatedly inflated until the stenosis is substantially eliminated. This procedure, as compared to heart surgery, is relatively noninvasive and typically involves a hospital stay of only a few days. The procedure is an important tool in the management of serious heart conditions.

A serious drawback to angioplasty procedures is the re-occurrence of the stenosis at the site of the angioplasty, or "restenosis". The clinical effects of angioplasty include endothelial denudation and vascular wall damage. In many cases, these injuries have been found to cause proliferation of the arterial smooth muscle cells and, it is believed, restenosis. Restenosis may occur in as many as 40% of patients that have undergone an angioplasty procedure. The FLIP molecules of the invention can be used to promote the viability of endothelial cells, thereby inhibiting the development of an adverse medical condition.

The invention also involves the use of the FLIP molecules for treating subjects who have primary or secondary pulmonary hypertension. Pulmonary hypertension as used herein means a right ventricular systolic or a pulmonary artery systolic pressure, at rest, of at least 20 mmHg. Pulmonary hypertension is measured using conventional procedures well-known to those of ordinary skill in the art. Pulmonary hypertension can have a variety of etiologies.

The invention also is useful for treating cardio-vascular or pulmonary vascular remodeling associated with vascular wall inflammation. Cardio-vascular remodeling can arise from numerous conditions, including acute trauma and chronic conditions affecting the cardio-vascular system. It can be associated, for example, in connection with cor-pulmonale, where the pulmonary vasculature as well as portions of the heart undergo changes involving luminal narrowing leading to a decreased ejection capacity of the right ventricle because of the greater pressure necessary to push blood through the narrow vessels. Pulmonary vascular remodeling is determined indirectly by echocardiogram or by right heart catheterization assessment of the associated pulmonary hypertension.

A subject, as used herein, refers to any mammal (preferably, a human) that may be susceptible to a condition associated with vascular wall inflammation and, optionally, a subject who exhibits elevated vascular levels of oxidized lipid (such as the conditions described above), provided that the mammal is otherwise free of symptoms calling for FLIP treatment. "Oxidized lipid", as used herein, refers to the oxidation products of lipids that are present in the vasculature. It has been reported that the endothelial cells play a role in the oxidation of lipid and that, in particular, these cells play a role in the oxidation of low density lipoprotein (LDL) to oxidized LDL. (See, e.g., R. Ross, *Nature* 362:801-809 (1993)). Preferred subjects are not otherwise being treated using viral vector gene therapy protocols.

A "FLIP molecule", as used herein, embraces both "FLIP nucleic acids" and "FLIP polypeptides" (discussed below). FLIP molecules are capable of preventing Fas ligand-mediated apoptosis in endothelial cells that express a Fas receptor. Accordingly, FLIP molecules are capable of reducing or preventing Fas ligand-mediated apoptosis of vascular endothelial cells in vivo and in vitro by preventing apoptosis in these cells.

A "FLIP nucleic acid", as used herein, refers to a nucleic acid molecule which: (1) has the sequence of SEQ. ID NO. 1 (the "Homo sapiens FLICE-like inhibitory protein long form mRNA, complete cds" having GenBank Accession No. U97074 human FLIP molecule) or hybridizes under stringent conditions to a cDNA that is transcribed into SEQ. ID NO. 1 and (2) codes for a FLIP polypeptide (i.e., a protein which inhibits Fas ligand-mediated apoptosis). The preferred FLIP nucleic acid has the nucleic acid sequence of SEQ. ID No. 1 (human FLIP-L) or SEQ. ID No. 3 (human FLIP-S). The FLIP nucleic acids of the invention also include homologs and alleles of the nucleic acids having the sequences of SEQ. ID. Nos. 1 or 3, as well as functionally equivalent fragments, variants, and analogs of the foregoing nucleic acids.

“Functionally equivalent”, in reference to a FLIP nucleic acid fragment, variant, or analog, refers to a nucleic acid that codes for a FLIP polypeptide that contains at least one “death effector domain” and that is capable of preventing Fas ligand-mediated apoptosis (e.g., of endothelial cells) in vivo or in vitro. In this manner, the FLIP molecules of the invention are capable of inhibiting vascular wall inflammation in vivo.

The term “isolated”, as used herein in reference to a nucleic acid molecule, means a nucleic acid sequence: (i) amplified in vitro by, for example, polymerase chain reaction (PCR); (ii) synthesized by, for example, chemical synthesis; (iii) recombinantly produced by cloning; or (iv) purified, as by cleavage and gel separation. The term “isolated”, as used herein in reference to a polypeptide (protein), means a polypeptide encoded by an isolated nucleic acid sequence, as well as polypeptides synthesized by, for example, chemical synthetic methods, and polypeptides separated from biological materials, and then purified using conventional protein analytical procedures.

Preferably, the FLIP nucleic acid has the nucleotide sequence of SEQ. ID NO 1, the nucleotide sequence encoding an “intact FLIP polypeptide”, i.e., the complete coding sequence of the gene encoding human FLIP-L. The intact human FLIP polypeptide (long form) contains the above-described “death effector domains” and “caspase-like subunits” as following: a DED I domain (amino acids 1 to 74); a DED II domain (amino acids 93 to 171); a caspase-like first subunit (amino acids 242 to 376); and a caspase-like second subunit (amino acids 377 to 480); the numbering is based upon that reported by M. Irmeler, et al., *Nature* 388: 190-195 (1997) for human FLIP-L.

The isolated FLIP nucleic acids of the invention also include nucleic acids encoding fragments of an intact FLIP. Preferably, the fragments are functional equivalents of the intact FLIP nucleic acid. For example, the FLIP nucleic acids may encode a fragment that contains one or more DED sequences, alone or coupled to one another and, optionally, further in combination with one or more caspase-like subunits as described above.

The above-described, functionally equivalent fragments of an intact FLIP polypeptide previously have not been described. Accordingly, one particular aspect of the invention relates to such FLIP polypeptides that are fragments of intact FLIP, nucleic acids encoding same, complements of said nucleic acids, antibodies that selectively bind to said polypeptides and that do not bind to intact FLIP polypeptide, vectors containing said nucleic acids, host cells containing said vectors, and methods for using the foregoing compositions.

In the preferred embodiments of the methods, the FLIP nucleic acid is selected from the group consisting of an intact FLIP nucleic acid (e.g., SEQ. ID NO. 1, SEQ. ID NO. 3), and any fragments of SEQ. ID Nos. 1 or 3 which contain one or more DED sequences, alone or coupled to one or more caspase-like subunits as described herein.

5 The FLIP nucleic acid is operatively coupled to a promoter that can express the FLIP polypeptide in a targeted cell (e.g., a vascular endothelial cell). More preferably, the FLIP nucleic acid is operatively coupled to a promoter that is not down regulated, directly or indirectly, by oxidized lipid. Exemplary promoters that can be used for this purpose include strong viral promoters such as CMV. Preferably, the nucleic acid is contained in an appropriate expression  
10 vector (e.g., adenoviral vector, modified adenoviral vector, retroviral vector, plasmid, liposome) to more efficiently genetically modify the targeted cell and achieve expression of multiple copies of the FLIP polypeptide in the target cell.

FLIP nucleic acids further embrace nucleic acid molecules which code for the FLIP polypeptides having the sequences of SEQ. ID Nos. 2 or 4 but which differ from the sequence of  
15 SEQ. ID Nos. 1 or 3, respectively, in codon sequence due to the degeneracy of the genetic code. The invention further embraces unique fragments (which may, or may not be "functional" with respect to encoding a FLIP protein) and complements of the foregoing nucleic acids, particularly, unique fragments of the FLIP nucleic acids. Such unique fragments can be used, for example, as probes in hybridization assays and as primers in a polymerase chain reaction (PCR) to generate  
20 the intact and fragment FLIP nucleic acids of the invention.

The FLIP nucleic acids of the invention can be identified by conventional techniques, e.g., by identifying nucleic acid sequences which code for FLIP polypeptides and which have the sequence of SEQ. ID Nos. 1 or 3 or which hybridize to a nucleic acid molecule having the sequence of SEQ. ID NOs. 1 or 3 under stringent conditions. The term "stringent conditions", as  
25 used herein, refers to parameters with which the art is familiar. More specifically, stringent conditions, as used herein, refer to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin, 2.5mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH 7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetraacetic acid. After hybridization,  
30 the membrane to which the DNA is transferred is washed at 2x SSC at room temperature and then at 0.1x SSC/0.1x SDS at 65°C.

There are other conditions, reagents, and so forth which can be used, which result in a



similar degree of stringency. The skilled artisan will be familiar with such conditions and, thus, they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of homologs and alleles of the FLIP nucleic acid of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for the expression of molecules, such as FLIP, can be isolated, following by isolation of the pertinent nucleic acid molecule and sequencing. In screening for FLIP nucleic acid sequences, a Southern blot may be performed using the foregoing conditions, together with a radioactive probe. After washing the membrane to which the DNA is finally transferred, the membrane can be placed against x-ray film to detect the radioactive signal.

In general, homologs and alleles typically will share at least 40% nucleotide identity with SEQ. ID. NOs. 1 or 3, or a cDNA that is transcribed into SEQ. ID Nos. 1 or 3; in some instances, will share at least 50% nucleotide identity; and in still other instances, will share at least 60% nucleotide identity. Watson-Crick complements of the foregoing nucleic acids are also embraced by the invention. The preferred homologs have at least 70% sequence homology to SEQ. ID. Nos. 1 or 3. More preferably the preferred homologs have at least 80% and most preferably at least 90% sequence homology to SEQ. ID. No. 1.

The invention also includes degenerate nucleic acids which include alternative codons to those present in the naturally occurring nucleic acid that codes for the human FLIP polypeptide. For example, serine residues are encoded by the codons TCA, AGT, TCC, TCG, TCT and AGC. Each of the six codons is equivalent for the purposes of encoding a serine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the serine-encoding nucleotide codons may be employed to direct the protein synthesis apparatus, in vitro or in vivo, to incorporate a serine residue. Similarly, nucleotide sequence triplets which encode other amino acid residues include, but are not limited to, CCA, CCC, CCG and CCT (proline codons); CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); ACA, ACC, ACG and ACT (threonine codons); AAC and AAT (asparagine codons); and ATA, ATC and ATT (isoleucine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the naturally occurring isolated nucleic acids in codon sequence due to the degeneracy of the genetic code.

The invention also provides isolated unique fragments of SEQ. ID NO.1 and SEQ. ID NO. 2, and complements of the foregoing FLIP nucleic acids. A unique fragment is one that is a

'signature' for the larger nucleic acid. It, for example, is long enough to assure that its precise sequence is not found in molecules outside of the FLIP gene. Unique fragments can be used as probes in Southern blot assays to identify family members or can be used in amplification assays such as those employing PCR. As known to those skilled in the art, large probes such as 200 base pair (BP) or more are preferred for certain uses such as Southern blots, while smaller fragments will be preferred for uses such as PCR. The fragments are also useful as probes for mRNA in Northern blot analysis. Unique fragments also can be used to produce fusion proteins for generating antibodies or for generating immunoassay components. Unique fragments are also useful for a variety of assays to determine the protein binding regions of the nucleic acid, such as gel shift assays and can be cloned into reporter constructs such as a chloramphenicol acetyl transferase (CAT) vector to determine the active promoter and enhancer regions. Likewise, unique fragments can be employed to produce fragments of the FLIP polypeptide, such as a "FRAGMENT 1" (described below) FLIP polypeptide, useful, for example, in inhibiting Fas ligand-mediated apoptosis in Fas ligand receptor-expressing cells (e.g., endothelial cells) that contact a Fas ligand or a Fas ligand-expressing cell. Complements of unique fragments further can be used as antisense molecules to inhibit the expression of the FLIP polypeptide, particularly for preparing in vitro cell and animal models of vascular wall inflammation.

As will be recognized by those skilled in the art, the size of the unique fragment will depend upon its conservancy in the genetic code. Thus, some regions of SEQ. ID. Nos. 1 or 3, will require longer segments to be unique while others will require only short segments, typically between 12 and 32 base pairs. Virtually any segment of SEQ. ID Nos. 1 or 3, that is 18 or more nucleotides in length will be unique. Those skilled in the art are well versed in methods for selecting such sequences, typically on the basis of the ability of the unique fragment to selectively distinguish the sequence of interest from other family members. Unique fragments of the FLIP polypeptides of the invention, nucleic acids encoding same, are a particularly preferred aspect of the invention. A comparison of the sequence of the fragment to those on known data bases typically is all that is necessary, although in vitro confirmatory hybridization and sequencing analysis optionally is performed. In particular, the FLIP molecules of the invention that are based upon unique combinations of the various domains of the FLIP molecule previously have not been described.

The FLIP nucleic acid, in one embodiment, is operably linked to a gene expression sequence which directs the expression of the FLIP nucleic acid within a eukaryotic cell. The

"gene expression sequence" is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the FLIP nucleic acid to which it is operably linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase,  $\beta$ -actin promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the simian virus, papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus (CMV), the long terminal repeats (LTR) of moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are modulated in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art and include the tetracycline-based, high-level gene expression systems described by Bujard, Gossen, and colleagues (Gossen, M. & Bujard, H., 1992, *Proc. Natl. Acad. Sci. USA*, 89:5547-5551; Gossen, M., et al., 1995, *Science*, 268:1766-1769. The preferred promoters for use in connection with the instant invention are those that are not down-regulated (directly or indirectly) by oxidized lipid.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined FLIP nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

Preferably, the FLIP nucleic acid of the invention is linked to a gene expression sequence which permits expression of the FLIP nucleic acid in an endothelial cell. More preferably, the gene expression sequence permits expression of the FLIP nucleic acid in a human vascular endothelial cell and does not permit expression of the FLIP nucleic acid in smooth muscle cells, hepatocytes and other Fas receptor-expressing cell types because it is undesirable to interfere with

the normal apoptosis of these cells. A sequence which permits expression of the FLIP nucleic acid in a human vascular endothelial cell is one which is selectively active in vascular endothelial cells and thereby causes the expression of the FLIP nucleic acid in these cells. The following promoters can be used to express the FLIP nucleic acid in human vascular endothelial cells: CMV, Tie2 gene promoter. Those of ordinary skill in the art will be able to easily identify alternative promoters that are capable of expressing a FLIP nucleic acid in a vascular endothelial cell.

The FLIP nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the transcription and/or translation of the FLIP coding sequence under the influence or control of the gene expression sequence. If it is desired that the FLIP sequence be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the FLIP sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the FLIP sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a FLIP nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that FLIP nucleic acid sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The FLIP nucleic acids of the invention can be delivered to the vascular endothelial cell alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating: (1) delivery of a FLIP molecule to a target cell and/or (2) uptake of a FLIP molecule by a target cell. Preferably, the vectors transport the FLIP molecule into the target cell with reduced degradation relative to the extent of degradation that would result in the absence of the vector. Optionally, a "targeting ligand" can be attached to the vector to selectively deliver the vector to a cell which expresses on its surface the cognate receptor for the targeting ligand. In this manner, the vector (containing a FLIP nucleic acid or a FLIP protein) can be selectively delivered to a vascular endothelial cell in, e.g., the arterial wall. Methodologies for targeting include conjugates, such as those described in U.S. Patent 5,391,723 to Priest. Another example of a well-known targeting vehicle is a liposome. Liposomes are commercially available from Gibco BRL. Numerous methods are published for making targeted liposomes. Preferably, the

FLIP molecules of the invention are targeted for delivery to an endothelial cell and, more preferably, a vascular endothelial cell.

In general, the vectors useful in the invention are divided into two classes: biological vectors and chemical/physical vectors. Biological vectors are useful for delivery/uptake of FLIP nucleic acids to/by a target cell. Chemical/physical vectors are useful for delivery/uptake of FLIP nucleic acids or FLIP proteins to/by a target cell.

Biological vectors include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences of the invention, and additional nucleic acid fragments (e.g., enhancers, promoters) which can be attached to the nucleic acid sequences of the invention. Viral vectors are a preferred type of biological vector and include, but are not limited to, nucleic acid sequences from the following viruses: adenovirus; adeno-associated virus; retrovirus, such as moloney murine leukemia virus; harvey murine sarcoma virus; murine mammary tumor virus; rouse sarcoma virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known in the art.

A particularly preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus is capable of infecting a wide range of cell types and species and can be engineered to be replication-deficient. It further has advantages, such as heat and lipid solvent stability, high transduction frequencies in cells of diverse lineages, including hemopoietic cells, and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion. The preparation of an adeno-associated virus containing a nucleic acid encoding the intact human FLIP is described in the Examples. This construct is designated "Adeno-FLIP" and contains a serotype 5 human replication defective adenovirus encoding the full-length human FLIP polypeptide from the CMV promoter/enhancer). Of course, other isoforms of the FLIP polypeptide coding sequences can be substituted for that described in the Examples to provide alternative constructs

that are useful for practicing the inventions disclosed herein.

Adeno-FLIP constructs can be constructed by subcloning a human FLIP cDNA, e.g., based upon the sequences for FLIP-L (Accession # U97074) or FLIP-S (Accession # U97075), downstream from an appropriate expression cassette (for example, the CMV promoter/enhancer) into the EcoRV site of the pCO1 vector containing the Ad5 adenoviral sequences required for homologous recombination. The resulting plasmid can then be linearized by restriction enzyme digestion and cotransfected in 293 cells with large ClaI fragment of the Ad5 d1324 viral DNA (Stratford-Perricaudet, L.D., et al., 1993, *J. Clin. Invest.* 90:626-630). The resulting replication-defective recombinant adenoviral constructs are then purified from isolated plaques. The viral preparations are typically purified by two CsCl gradient centrifugations, dialyzed against buffer containing 10 mM Tris-Cl pH 7.5, 1 mM MgCl<sub>2</sub> and 135 mM NaCl and stored at -80°C in 10% glycerol. Viral titer is typically determined by plaque assay on 293 cells (Graham, F.L., and A.J. van der Eb, 1973, *Virology* 52:456-463) and expressed as plaque forming units (pfu) per ml.

Other adeno-FLIP constructs can be constructed by substituting an inducible promoter (e.g., a tetracycline-based cassette) for the constitutive promoter (e.g. the CMV promoter/enhancer). Preferred tetracycline-based inducible promoter systems include the Tet-Off™ & Tet-On™ Gene Expression Systems available from Clontech, Palo Alto, CA. Briefly, Tet-Off and Tet-On Gene Expression Systems allow high-level, regulated gene expression in response to varying concentrations of tetracycline (Tc) or Tc derivatives such as doxycycline (Dox). In the Tet-Off System, gene expression is turned on in the absence of Tc or Dox. In contrast, gene expression is activated in the Tet-On System in the presence of Tc or Dox. The Tet Expression Systems are based on two regulatory elements derived from the tetracycline-resistance operon of the *E. coli* Tn10 transposon--the tetracycline repressor protein (TetR) and the tetracycline operator sequence (tetO) to which TetR binds. The gene to be expressed (e.g., FLIP-L: SEQ ID NO:1, FLIP-S: SEQ ID NO:3, FLIP-homologs, etc.) is cloned into the pTRE "response" plasmid, which contains the PhCMV\*-1 promoter upstream of a multiple cloning site (MCS). PhCMV\*-1 is a compound promoter consisting of the tetracycline-responsive element (TRE), which contains seven copies of tetO, and the minimal immediate early promoter of cytomegalovirus (PminCMV). The second key component of the system is a "regulator" plasmid which expresses a hybrid protein known as the Tc-controlled transactivator (tTA). tTA is encoded by pTet-Off and is a fusion of the wild-type TetR to the VP16 activation domain (AD) of herpes simplex virus. tTA binds the tetO sequences which

brings the VP16 activation domain into close proximity with the PhCMV\*-1--and thereby activates transcription--in the absence of Tc. Thus, as Tc is added to the culture medium, transcription is turned off in a dose-dependent manner. The Tet-On System is based on the "reverse" TetR (rTetR), which differs from the wild-type TetR by four amino acid changes  
5 (Gossen, M., et al., 1995, *Science*, 268:1766-1769). When fused to the VP16 AD, rTetR creates a "reverse" tTA (rtTA) that activates transcription in the presence of Tc or Dox. As described in the examples, the Tet-On System was utilized to generate the tetracycline-responsive FLIP adenoviral constructs.

In general, other preferred viral vectors are based on non-cytopathic eukaryotic viruses  
10 in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Adenoviruses and retroviruses have been approved for human gene therapy trials. In general, the retroviruses are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable  
15 of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue  
20 culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., "Gene Transfer and Expression, A Laboratory Manual," W.H. Freeman C.O., New York (1990) and Murry, E.J. Ed. "Methods in Molecular Biology," vol. 7, Humana Press, Inc., Clifton, New Jersey (1991).

Another preferred retroviral vector is the vector derived from the moloney murine  
25 leukemia virus, as described in Nabel, E.G., et al., *Science*, v. 249, p. 1285-1288 (1990). These vectors reportedly were effective for the delivery of genes to all three layers of the arterial wall, including the intima, which is comprised of endothelial cells. Other preferred vectors are disclosed in Flugelman, et al., *Circulation*, v. 85, p. 1110-1117 (1992). Alternatively, naked FLIP nucleic acids or plasmids containing FLIP nucleic acids can be used in place of viral vectors to  
30 genetically modify the target cells to express functional FLIP polypeptides.

In addition to the biological vectors, chemical/physical vectors may be used to deliver a FLIP molecule to a target cell and facilitate uptake thereby. As used herein, a "chemical/physical

vector" refers to a natural or synthetic molecule, other than those derived from bacteriological or viral sources, capable of delivering the isolated FLIP molecule to a cell.

A preferred chemical/physical vector of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vessels which are useful as a delivery vector in vivo or in vitro. It has been shown that large unilamellar vessels (LUV), which range in size from 0.2 - 4.0  $\mu$  can encapsulate large macromolecules. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.*, v. 6, p. 77 (1981)). In order for a liposome to be an efficient gene transfer vector, one or more of the following characteristics should be present: (1) encapsulation of the gene of interest at high efficiency with retention of biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information.

Liposomes may be targeted to a particular tissue, such as the vascular cell wall, by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be useful for targeting a liposome to the vascular wall include, but are not limited to the viral coat protein of the Hemagglutinating virus of Japan. Additionally, the vector may be coupled to a nuclear targeting peptide, which will direct the FLIP nucleic acid to the nucleus of the host cell.

Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN<sup>TM</sup> and LIPOFECTACE<sup>TM</sup>, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications. Liposomes also have been reviewed by Gregoriadis, G. in *Trends in Biotechnology*, V. 3, p. 235-241 (1985).

In one particular embodiment, the preferred vehicle is a biocompatible micro particle or implant that is suitable for implantation into the mammalian recipient. Exemplary bioerodible implants that are useful in accordance with this method are described in PCT International application no. PCT/US/03307 (Publication No. WO 95/24929, entitled "Polymeric Gene Delivery System", claiming priority to U.S. patent application serial no. 213,668, filed March 15,



1994). PCT/US/0307 describes a biocompatible, preferably biodegradable polymeric matrix for containing an exogenous gene under the control of an appropriate promotor. The polymeric matrix is used to achieve sustained release of the exogenous gene in the patient. In accordance with the instant invention, the FLIP nucleic acids described herein are encapsulated or dispersed within the biocompatible, preferably biodegradable polymeric matrix disclosed in PCT/US/03307. The polymeric matrix preferably is in the form of a micro particle such as a micro sphere (wherein the FLIP nucleic acid is dispersed throughout a solid polymeric matrix) or a microcapsule (wherein the FLIP nucleic acid is stored in the core of a polymeric shell). Other forms of the polymeric matrix for containing the FLIP nucleic acid include films, coatings, gels, implants, and stents. The size and composition of the polymeric matrix device is selected to result in favorable release kinetics in the tissue into which the matrix device is implanted. The size of the polymeric matrix device further is selected according to the method of delivery which is to be used, typically injection into a tissue or administration of a suspension by aerosol into the nasal and/or pulmonary areas. The polymeric matrix composition can be selected to have both favorable degradation rates and also to be formed of a material which is bioadhesive, to further increase the effectiveness of transfer when the device is administered to a vascular surface. The matrix composition also can be selected not to degrade, but rather, to release by diffusion over an extended period of time.

Both non-biodegradable and biodegradable polymeric matrices can be used to deliver the FLIP nucleic acids of the invention to the subject. Biodegradable matrices are preferred. Such polymers may be natural or synthetic polymers. Synthetic polymers are preferred. The polymer is selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve months is most desirable. The polymer optionally is in the form of a hydrogel that can absorb up to about 90% of its weight in water and further, optionally is cross-linked with multi-valent ions or other polymers.

In general, the FLIP nucleic acids of the invention are delivered using the bioerodible implant by way of diffusion, or more preferably, by degradation of the polymeric matrix. Exemplary synthetic polymers which can be used to form the biodegradable delivery system include: polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, poly-vinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes and co-polymers

thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxyl ethyl cellulose, cellulose triacetate, cellulose sulphate sodium salt, poly(methyl methacrylate),  
5 poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl alcohols), polyvinyl acetate, poly vinyl chloride,  
10 polystyrene and polyvinylpyrrolidone.

Examples of non-biodegradable polymers include ethylene vinyl acetate, poly(meth)acrylic acid, polyamides, copolymers and mixtures thereof.

Examples of biodegradable polymers include synthetic polymers such as polymers of lactic  
15 acid and glycolic acid, polyanhydrides, poly(ortho)esters, polyurethanes, poly(butic acid), poly(valeric acid), and poly(lactide-cocaprolactone), and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, chemical derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art), albumin and other  
20 hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. In general, these materials degrade either by enzymatic hydrolysis or exposure to water *in vivo*, by surface or bulk erosion.

Bioadhesive polymers of particular interest include bioerodible hydrogels described by H.S. Sawhney, C.P. Pathak and J.A. Hubell in *Macromolecules*, 1993, 26, 581-587, the teachings  
25 of which are incorporated herein, polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, poly(methyl methacrylates), poly(ethyl methacrylates), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate). Thus, the  
30 invention provides a composition of the above-described FLIP molecules for use as a medicament, methods for preparing the medicament and methods for the sustained release of the medicament *in vivo*. In the preferred embodiments, the FLIP nucleic acid has the nucleic acid

sequence of SEQ. ID NOs. 1 or 3 or a functionally-equivalent fragment of SEQ. ID Nos. 1 or 3, such as a nucleic acid encoding a "FRAGMENT 1" FLIP polypeptide or a nucleic acid encoding a "FRAGMENT 2" FLIP polypeptide as described herein. Preferably, the FLIP nucleic acid is operably linked to a gene expression sequence to permit expression of the FLIP polypeptide in the target cell. The preferred FLIP polypeptide has the amino acid sequence of SEQ. ID NOs. 2 or 4 or a functionally equivalent fragment of SEQ. ID NOs. 2 or 4 that contains one or more "death effector domains" and, optionally, one or more caspase-like subunits. The sequences for these various domains, as well as their binding properties, are known to those of ordinary skill in the art.

Compaction agents also can be used alone, or in combination with, a biological or chemical/physical vector of the invention. A "compaction agent", as used herein, refers to an agent, such as a histone, that neutralizes the negative charges on the nucleic acid and thereby permits compaction of the nucleic acid into a fine granule. Compaction of the nucleic acid facilitates the uptake of the nucleic acid by the target cell. The compaction agents can be used alone, i.e., to deliver the isolated FLIP nucleic acid in a form that is more efficiently taken up by the cell or, more preferably, in combination with one or more of the above-described vectors.

Other exemplary compositions that can be used to facilitate uptake by a target cell of the FLIP nucleic acids include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a FLIP nucleic acid into a preselected location within the target cell chromosome).

The FLIP nucleic acids code for a FLIP polypeptide. As used herein, a "FLIP polypeptide" refers to a polypeptide that is coded for by a FLIP nucleic acid and that binds to a "death effector domain" (DED) but does not activate downstream proteases. The FLIP polypeptides inhibit Fas ligand-mediated apoptosis. Preferably, the FLIP polypeptide inhibits Fas ligand-mediated apoptosis in an endothelial cell and, more particularly, inhibits apoptosis in a vascular endothelial cell. Accordingly, FLIP polypeptides are useful for inhibiting Fas ligand-mediated vascular endothelial cell apoptosis in vivo and in vitro. The preferred FLIP polypeptides of the invention have the amino acid sequence of SEQ. ID NOs. 2 or 4 or are functionally equivalent fragments of SEQ. ID Nos. 2 or 4. FLIP polypeptides further embrace functionally equivalent variants, and analogs of SEQ. ID NO. 2 or 4, provided that the fragments, variants, and analogs are capable of binding to a death effector domain and, optionally, inhibiting Fas ligand-mediated apoptosis.

The invention also embraces proteins and peptides coded for by any of the foregoing FLIP nucleic acids.

A "functionally equivalent variant" of SEQ. ID NO. 2 or 4 binds to a death domain and, optionally, inhibits Fas ligand-induced apoptosis. In particular, a functionally equivalent variant of SEQ. ID NO. 2 or 4 is capable of inhibiting such apoptosis in an endothelial cell (e.g., a vascular endothelial cell) in vitro or in vivo. An in vitro apoptosis assay (see, e.g., the apoptosis assay provided in the Examples) can be used as a screening assay to measure the ability of a polypeptide to inhibit Fas ligand-mediated apoptosis in a vascular endothelial cell in vitro and is predictive of the ability of the polypeptide to inhibit apoptosis of vascular endothelial cells in vivo. Alternatively or additionally, binding assays can be used to select such functionally equivalent variants, e.g., by selecting variant that bind to a death domain in an in vitro assay. Such binding assays to select protein-type binding cognates are known to those of ordinary skill in the art. Exemplary "functionally equivalent variants" of SEQ. ID. Nos. 2 or 4 includes fragments of SEQ. ID. Nos. 2 or 4, as well as polypeptide analogs of SEQ. ID. Nos. 2 or 4 which contain conservative amino acid substitutions, provided that the polypeptide variants and analogs are capable of binding to a death effector domain and, preferably, inhibiting Fas ligand-mediated apoptosis of vascular endothelial cells.

The preferred FLIP nucleic acids of the invention encode the FLIP having the amino acid sequence of SEQ. ID NO. 2, the complete coding sequence of the gene encoding the human FLIP. This "intact" human FLIP polypeptide contains two death effector domains and four caspase-like domains as described above.

The invention also embraces compositions containing and methods for using "functionally equivalent fragments" of the FLIP polypeptide, namely, "FRAGMENT 1 polypeptides" and "FRAGMENT 2 polypeptides", and so forth as described herein. Preferably, such polypeptides are fragments of SEQ. ID Nos. 2 or 4. No prior use for the FRAGMENT 1 polypeptides and FRAGMENT 2 polypeptides disclosed here has been proposed. Accordingly, one particular aspect of the invention relates to such FRAGMENT 1 polypeptides, nucleic acids encoding same, complements of said nucleic acids, vectors containing said nucleic acids, host cells containing said vectors, and methods for using the foregoing compositions. Alternative embodiments include FLIP polypeptides that are identical in amino acid sequence to SEQ. ID Nos. 2 or 4 and fragments of SEQ. ID Nos. 2 or 4, but which differ from SEQ. ID Nos. 2 or 4 in having one or more amino acid substitutions in the caspase-like domain proteolytic cleavage site, i.e., amino

acid Asp 376 in SEQ. ID NO. 1. See also, Irmmler et al., *Nature supra.*, which describes the cleavage of FLIP-L at Asp 376 to yield a molecule that reportedly binds with greater affinity to FLICE.

It will be appreciated by those skilled in the art that various modifications of the FLIP polypeptide having the sequence of SEQ. ID. Nos. 2 or 4 or functionally equivalent fragments of SEQ. ID NO.2 can be made without departing from the essential nature of the invention. Accordingly, it is intended that polypeptides which have the amino acid sequence of SEQ. ID NO. 2 but which include conservative substitutions are embraced within the instant invention. As used herein, "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the polypeptide in which the amino acid substitution is made. Conservative substitutions of amino acids include substitutions made amongst amino acids with the following groups: (1) M,I,L,V; (2) F,Y,W; (3) K,R,H; (4) A,G; (5) S,T; (6) Q,N; and, (7) E,D. Fusion proteins, in which a peptide of the invention is coupled to a solid support (such as a polymeric bead), a carrier molecule (such as keyhole limpet hemocyanin), or a reporter group (such as radiolabel or other tag), also are embraced within the invention.

When used therapeutically, the isolated FLIP molecules of the invention are administered in therapeutically effective amounts. In general, a therapeutically effective amount means that amount necessary to delay the onset of, inhibit the progression of, or halt altogether the particular condition being treated. Generally, a therapeutically effective amount will vary with the subject's age, condition, and sex, as well as the nature and extent of the disease in the subject, all of which can be determined by one of ordinary skill in the art. The dosage may be adjusted by the individual physician or veterinarian, particularly in the event of any complication. A therapeutically effective amount typically varies from 0.01 mg/kg to about 1000 mg/kg, preferably from about 0.1 mg/kg to about 200 mg/kg, and most preferably from about 0.2 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or more days.

The therapeutically effective amount of the isolated FLIP molecule is that amount effective to inhibit vascular wall inflammation as determined by, for example, standard tests known in the art. It is believed that the FLIP molecules inhibit apoptosis in the target cells by interfering with the Fas ligand-Fas receptor signaling pathway. For example, TUNEL staining, and the appearance of condensed chromatin and other morphological features characteristic of apoptosis in electron micrographs can be used to assess apoptosis in vascular endothelial and other cell types.

Optionally, the isolated FLIP molecule is administered to the subject in combination with a method for treating an arteriosclerotic condition. An arteriosclerotic condition, as used herein, is a term of art that refers to classical atherosclerosis, transplant arteriosclerosis, accelerated atherosclerosis, atherosclerotic lesions and other physiological conditions characterized by undesirable vascular wall inflammation. See, e.g., *Harrisons, Principles of Internal Medicine* (McGraw Hill, Inc., New York) for a more detailed description of these conditions. The method for treating an arteriosclerotic condition may be a surgical method, an agent for treating restenosis, a method involving a drug therapy (e.g., gene therapy) or a combination of the foregoing.

Surgical methods for treating an arteriosclerotic condition include procedures such as bypass surgery, atherectomy, laser procedures, ultrasonic procedures, and balloon angioplasty. In a preferred embodiment of the invention, the isolated FLIP molecule is administered to a subject in combination with a balloon angioplasty procedure. A balloon angioplasty procedure involves inserting a catheter having a deflated balloon into an artery. The deflated balloon is positioned in proximity to the atherosclerotic plaque and is inflated such that the plaque is compressed against the arterial wall. As a result, the layer of endothelial cells on the surface of the artery is disrupted. The isolated FLIP molecule is attached to the balloon angioplasty catheter in a manner which permits release of the isolated FLIP molecule to the remaining endothelium at the site of the atherosclerotic plaque. The isolated FLIP molecule may be attached to the balloon angioplasty catheter in accordance with standard procedures known in the art. For example, the isolated FLIP molecule may be stored in a compartment of the balloon angioplasty catheter until the balloon is inflated, at which point it is released into the local environment. Alternatively, the isolated FLIP molecule may be impregnated on the balloon surface, such that it contacts the cells of the arterial wall as the balloon is inflated. The FLIP molecule also may be delivered in a perforated balloon catheter such as those disclosed in Flugelman, et al., *Circulation*, v. 85, p. 1110-1117 (1992). See, also, e.g., published PCT Patent Application WO 95/23161, for an exemplary procedure for attaching a therapeutic protein to a balloon angioplasty catheter. This procedure can be modified using no more than routine experimentation to attach a therapeutic nucleic acid or polypeptide to the balloon angioplasty catheter.

Additionally, the FLIP molecule may be administered with an agent for treating or preventing clinically significant restenosis, which often occurs following balloon angioplasty procedures. Restenosis is narrowing of the artery which occurs in 25% to 50% of patients within

6 months of an angioplasty procedure. Although restenosis was originally believed to be due completely to local tissue growth, recent findings have suggested that it may be due to a combination of tissue growth and vessel constriction. Moreover, although intravascular stents are being widely used to prevent vessel constriction, such stents induce tissue growth and, thereby, promote restenosis. Accordingly, the delivery of an anti-atherosclerotic, such as the FLIP molecules of the invention, is believed to be useful for treating vascular remodeling, in general, and in-stent restenosis, in particular, through its ability to promote endothelial cell viability and preserve the integrity of the endothelium.

A preferred agent for preventing restenosis, in combination with the FLIP molecule, is a stent. Stents are discussed in a review article by Topol, E. J., the contents of which are hereby incorporated by reference (Topol, E. J., *N. E. J. Med.* 331: 539-41 (1994)). Stents include, for example, the Gianturco-Roubin stent and the Palmaz-Schatz stent.

The arteriosclerotic conditions also can be treated by a nonsurgical method such as a drug therapy. Many drugs have been used to treat various aspects of an arteriosclerotic condition. For example, drugs have been used to treat physiological events, such as hypertension and excessive cholesterol accumulation, which are believed to contribute to the formation of atherosclerotic plaques. Other drugs influence the site of injury by breaking up or reducing the size of atherosclerotic plaques, and/or increasing the strength of the arterial wall. The isolated FLIP molecule may be administered in conjunction with either or a combination of drugs which inhibit the physiological events contributing to arteriosclerosis or drugs which function directly to reduce the local site of injury associated with atherosclerosis.

Drug therapies which have been found to be useful in treating the physiological events contributing to the development of the atherosclerotic injury, include, but are not limited to, the following drugs: diuretics, antiadrenergic agents, vasodilators, calcium channel antagonists, HMG-CoA reductase inhibitors, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II antagonists, and clot dissolvers.

Diuretics include thiazides, e.g., hydrochlorothiazide; loop acting diuretics, e.g., furosemide; potassium-sparing, e.g., spironolactone, triamterene, and amiloride.

Antiadrenergic agents include clonidine; guanabenz; guanfacine; methyl dopa; trimethopazine; Rauwolfia alkaloids, e.g., reserpine; guanethidine; guanadrel; phentolamine; phenoxybenzamine; prazosin; terazosin; propranolol; metoprolol; nadolol; atenolol; timolol; timolol; acebutolol; and labetalol.

Vasodilators include hydralazine; minoxidil; diazoxide; and nitroprusside.

Calcium channel antagonists include nisadipine; diltiazem; and verapamil.

Angiotensin II antagonists are compounds which interfere with the activity of angiotensin II by binding to angiotensin II receptors and interfering with its activity. Angiotensin II antagonists are well known and include peptide compounds and non-peptide compounds. Most angiotensin II antagonists are slightly modified congeners in which agonist activity is attenuated by replacement of phenylalanine in position 8 with some other amino acid; stability can be enhanced by other replacements that slow degeneration *in vivo*. Examples of angiotensin II antagonists include: peptidic compounds (e.g., saralasin, [(San<sup>1</sup>)(Val<sup>5</sup>)(Ala<sup>8</sup>)] angiotensin -(1-8) octapeptide and related analogs); N-substituted imidazole-2-one (US Patent Number 5,087,634); imidazole acetate derivatives including 2-N-butyl-4-chloro-1-(2-chlorobenzyl) imidazole-5-acetic acid (see Long et al., *J. Pharmacol. Exp. Ther.* 247(1), 1-7 (1988)); 4, 5, 6, 7-tetrahydro-1H-imidazo [4, 5-c] pyridine-6-carboxylic acid and analog derivatives (US Patent Number 4,816,463); N2-tetrazole beta-glucuronide analogs (US Patent Number 5,085,992); substituted pyrroles, pyrazoles, and triazoles (US Patent Number 5,081,127); phenol and heterocyclic derivatives such as 1, 3-imidazoles (US Patent Number 5,073,566); imidazo-fused 7-member ring heterocycles (US Patent Number 5,064,825); peptides (e.g., US Patent Number 4,772,684); antibodies to angiotensin II (e.g., US Patent Number 4,302,386); and aralkyl imidazole compounds such as biphenyl-methyl substituted imidazoles (e.g., EP Number 253,310, January 20, 1988); ES8891 (N-morpholinoacetyl-(-1-naphthyl)-L-alanyl-(4, thiazolyl)-L-alanyl (35, 45)-4-amino-3-hydroxy-5-cyclo-hexapentanoyl-N-hexylamide, Sankyo Company, Ltd., Tokyo, Japan); SKF108566 (E-alpha-2-[2-butyl-1-(carboxy phenyl) methyl] 1H-imidazole-5-yl[methylane]-2-thiophenepropanoic acid, Smith Kline Beecham Pharmaceuticals, PA); Losartan (DUP753/MK954, DuPont Merck Pharmaceutical Company); Remikirin (RO42-5892, F. Hoffman LaRoche AG); A<sub>2</sub> agonists (Marion Merrill Dow) and certain non-peptide heterocycles (G.D.Searle and Company).

ACE, is an enzyme which catalyzes the conversion of angiotensin I to angiotensin II. ACE inhibitors include amino acids and derivatives thereof, peptides, including di and tri peptides and antibodies to ACE which intervene in the renin-angiotensin system by inhibiting the activity of ACE, thereby reducing or eliminating the formation of pressor substance angiotensin II. ACE inhibitors have been used medically to treat hypertension, congestive heart failure, myocardial infarction and renal disease. Classes of compounds known to be useful as ACE inhibitors include



acylmercapto and mercaptoalkanoyl prolines such as captopril (US Patent Number 4,105,776) and zofenopril (US Patent Number 4,316,906), carboxyalkyl dipeptides such as enalapril (US Patent Number 4,374,829), lisinopril (US Patent Number 4,374,829), quinapril (US Patent Number 4,344,949), ramipril (US Patent Number 4,587,258), and perindopril (US Patent Number 4,508,729), carboxyalkyl dipeptide mimics such as cilazapril (US Patent Number 4,512,924) and benazapril (US Patent Number 4,410,520), phosphinylalkanoyl prolines such as fosinopril (US Patent Number 4,337,201) andtrandolopril.

Renin inhibitors are compounds which interfere with the activity of renin. Renin inhibitors include amino acids and derivatives thereof, peptides and derivatives thereof, and antibodies to renin. Examples of renin inhibitors that are the subject of United States patents are as follows: urea derivatives of peptides (US Patent Number 5,116,835); amino acids connected by nonpeptide bonds (US Patent Number 5,114,937); di and tri peptide derivatives (US Patent Number 5,106,835); amino acids and derivatives thereof (US Patent Numbers 5,104,869 and 5,095,119); diol sulfonamides and sulfinyls (US Patent Number 5,098,924); modified peptides (US Patent Number 5,095,006); peptidyl beta-aminoacyl aminodiol carbamates (US Patent Number 5,089,471); pyroimidazolones (US Patent Number 5,075,451); fluorine and chlorine statine or statone containing peptides (US Patent Number 5,066,643); peptidyl amino diols (US Patent Numbers 5,063,208 and 4,845,079); N-morpholino derivatives (US Patent Number 5,055,466); pepstatin derivatives (US Patent Number 4,980,283); N-heterocyclic alcohols (US Patent Number 4,885,292); monoclonal antibodies to renin (US Patent Number 4,780,401); and a variety of other peptides and analogs thereof (US Patent Numbers 5,071,837, 5,064,965, 5,063,207, 5,036,054, 5,036,053, 5,034,512, and 4,894,437).

Drugs which are clot dissolvers include thrombolytic agents which have been used in the treatment of acute venous thromboembolism and pulmonary emboli and are well known in the art (e.g. see Hennekens et al, *J Am Coll Cardiol*; v. 25 (7 supp), p. 18S-22S (1995); Holmes, et al, *J Am Coll Cardiol*; v.25 (7 suppl), p. 10S-17S (1995)). Thrombolytic agents include, for example, direct acting agents such as streptokinase and urokinase, and second generation agents such as tissue plasminogen activator (tPA).

Drug therapies which influence the site of injury include any drug which contributes to the reduction of an atherosclerotic plaque or to the strengthening of the arterial wall in the local area of injury. Drugs which help to contribute to the reduction of the plaque include cytostatic molecules and antisense agents to cell cycle regulatory molecules. Other drugs which contribute

to the strengthening of the arterial wall include drugs which promote endothelial cell proliferation and function, such as cytokines.

In an embodiment of the invention, the isolated FLIP molecule is administered to a subject in combination with a cytostatic molecule for inhibiting excessive vascular smooth muscle cell proliferation. The cytostatic molecule is an agent (e.g., a nucleic acid, a protein) that suppresses cell growth and/or proliferation of vascular smooth muscle cells. Other agents and therapeutic methods for inhibiting excessive vascular smooth muscle cell proliferation are described in U.S. Serial No. 08/810,453, the contents of which are incorporated entirety herein by reference. A preferred cytostatic molecule is one which inhibits the growth and/or proliferation of vascular smooth muscle cells and includes the growth arrest homeobox molecule (GAX). The GAX molecule is described in published PCT Application WO95/23161. Another preferred cytostatic molecule is GATA-6 (preferably human GATA-6 as described in E. Suzuki, et al., 1996, *Genomics* 38:283-290). Other cytostatic molecules that are active with respect to vascular smooth muscle cells include the retinoblastoma protein (pRB), and cyclic kinase inhibitors, such as p21 and NO donors (Mooradian et al., *J. Cardiovasc. Pharmacol.* 25: 674-8 (1995)).

In another embodiment of the invention, the isolated FLIP molecule may be administered to a subject in combination with an antisense oligonucleotide that is targeted to vascular smooth muscle cells and that selectively hybridizes to cell cycle regulatory molecules, such as c-myc, cdc2, cdk2, PCNA, and c-myc under physiological conditions. Such antisense oligonucleotides can function as cytostatic or cytotoxic agent, depending upon the relative amounts of the antisense oligonucleotides that are delivered to the vascular smooth muscle cell and the importance of the particularly targeted cell cycle regulatory molecule to cell growth, proliferation and survival.

Certain cytokines function to strengthen the arterial wall by promoting endothelial cell proliferation. Cytokines which promote endothelial cell proliferation include, but are not limited, to the following: vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and acidic fibroblast growth factor (aFGF). Substances that stimulate the proliferation or migration of normal endothelial cells include factors which are associated with the vascularization of tumors and substances which inhibit angiogenesis. Such substances include transforming growth factor beta (TGF $\beta$ ), tumor necrosis factor alpha (TNF $\alpha$ ), human platelet factor 4 (PF4), and alpha interferon ( $\alpha$ INF); factors which suppress cell migration, such as proteinase inhibitors, tissue inhibitors of metalloproteinase (TIMP-1 and TIMP-2); and other

proteins such as protamine which has demonstrated angiostatic properties.

The above-described drug therapies are well known to those of ordinary skill in the art and are administered by modes known to those of skill in the art. The drug therapies are administered in amounts which are effective to achieve the physiological goals (to prevent or reduce the physiological consequences of atherosclerosis), in combination with the isolated FLIP molecule  
5 of the invention. Thus, it is contemplated that the drug therapies may be administered in amounts which are not capable of preventing or reducing the physiological consequences of atherosclerosis when the drug therapies are administered alone but which are capable of preventing or reducing the physiological consequences of atherosclerosis when administered in combination with the  
10 isolated FLIP molecules of the invention.

The isolated FLIP molecule may be administered alone or in combination with the above-described drug therapies as part of a pharmaceutical composition. Such a pharmaceutical composition may include the isolated FLIP molecule in combination with any standard physiologically and/or pharmaceutically acceptable carriers which are known in the art. The  
15 compositions should be sterile and contain a therapeutically effective amount of the isolated FLIP molecule in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration into a human or other animal. The term "carrier" denotes an organic or inorganic ingredient, natural  
20 or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy. Pharmaceutically acceptable further means a non-toxic material that is compatible with a  
25 biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

Compositions suitable for parenteral administration conveniently comprise a sterile  
30 aqueous preparation of the FLIP molecules, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation

also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulations suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular drug selected, the severity of the condition being treated, and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, interdermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis. They could, however, be preferred in emergency situations. Oral administration will be preferred for prophylactic treatment because of the convenience to the patient as well as the dosing schedule.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the FLIP molecules into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the FLIP molecules into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the FLIP molecule. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the FLIP molecules

described above, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include the above-described polymeric systems, as well as polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono- di- and tri-glycerides; hydrogel release systems; systatic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which an agent of the invention is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

Use of a long-term sustained release implant may be particularly suitable for treatment of chronic conditions. Long-term release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

The isolated FLIP molecule may be administered alone or in combination with the above-described drug therapies by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intra-cavity, subcutaneous, or transdermal. When using the isolated FLIP molecule of the invention, direct administration to the vessel injury site, such as by administration in conjunction with a balloon angioplasty catheter, is preferred.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution,

Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

5 In general, the FLIP nucleic acids can be administered to the subject (any mammalian recipient) using the same modes of administration that currently are used for gene therapy in humans (e.g., adenovirus-mediated gene therapy). Preferably, the FLIP nucleic acid (contained in, or associated with, an appropriate vector) is administered to the mammalian recipient by balloon angioplasty catheter (described above) or intra-vascular injection. Numerous transfection and transduction techniques as well as appropriate expression vectors are well known to those of  
10 ordinary skill in the art, some of which are described in PCT application WO95/00654.

As an illustrative example, a vector containing a FLIP nucleic acid is delivered to a site of vascular injury in a subject who is a candidate for such gene therapy. Then, the vector genetically modifies the vascular endothelial cells in vivo with DNA (RNA) encoding a FLIP polypeptide  
15 of the invention. Such genetically modified vascular endothelial cells are expected to inhibit Fas ligand-mediated apoptosis of vascular endothelial cells in vivo.

Another aspect of the invention includes a screening assay method for determining whether a putative therapeutic agent modulates Fas ligand-mediated apoptosis of endothelial cells. The method involves (1) contacting a cell that expresses Fas and FLIP with an oxidized lipid (e.g.,  
20 oxidized LDL) and a putative inhibitor under conditions wherein the cell is capable of undergoing Fas ligand-induced apoptosis (e.g., conditions such as adding a Fas ligand to induce apoptosis or, alternatively, an antibody that binds to Fas and mimics Fas ligand binding to its receptor); (2) determining whether the cell undergoes Fas ligand-induced apoptosis in the presence of the putative inhibitor; and (3) selecting the putative inhibitor that inhibits or prevents Fas ligand-  
25 induced apoptosis as an agent that inhibits the development or progression of atherosclerotic lesions. The putative inhibitor may be a component of a combinatorial library or peptide library and the assay is used to identify synthetic molecules that bind to the DED.

Oxidized LDL for use in the screening assay is prepared by oxidizing LDL either chemically, for example, by incubating LDL at 37°C for 24 hours, at about 0.2 mg protein/ml in  
30 PBS with about 20  $\mu$ M  $\text{CuSO}_4$  or via a cell mediated process, e.g., by incubating LDL in the presence of endothelial cells at 37° C for 24 hours, at a concentration of about 0.07 - 0.1 mg protein/ml, in serum free medium containing 12  $\mu$ M  $\text{CuSO}_4$ . An increase in the number of cells

that survive indicates that the putative therapeutic agent inhibits oxidized lipid-mediated down regulation (direct or indirect) of FLIP expression in the cell. The assay optionally includes one or more negative controls, e.g., cells of the same cell type which have not received a FLIP molecule but which have been exposed to Fas ligand. In one embodiment of the invention the method also involves the step of contacting the FLIP molecule with a detection reagent that selectively binds to the FLIP molecule to detect or measure the amount of the FLIP molecule in the "test" cell. The FLIP molecule may optionally be isolated from the vascular endothelial or other cell prior to contacting the isolated FLIP molecule with the detection reagent. When the FLIP molecule is a FLIP mRNA or cDNA, the detection reagent can be a nucleic acid that selectively hybridizes to the FLIP mRNA or cDNA. According to this embodiment, the "test" cell is contacted with the detection reagent under conditions that permit selective hybridization of the nucleic acid to the FLIP mRNA or cDNA. The preferred nucleic acid for this embodiment is a nucleic acid sequence having SEQ. ID. No. 1 or a functionally equivalent fragment thereof. Alternatively, the FLIP molecule that is being assayed can be a FLIP polypeptide and the detection reagent can be an antibody that selectively binds to the FLIP protein. The FLIP polypeptide can be contacted with the detection reagent under conditions that permit selective binding of a FLIP antibody to the FLIP polypeptide.

Alternatively, the FLIP nucleic acid of the invention can be used to prepare a non-human transgenic animal that can be used, for example, as an animal model (e.g., a FLIP knockout animal) of excessive smooth muscle cell proliferation. A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats. Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc. Transgenic animals having a particular property associated with a particular disease can be used to study the affects of a variety of drugs and treatment methods on the disease, and thus serve as genetic models for the study of a number of human diseases. The invention, therefore, contemplates the use of FLIP knockout and transgenic animals as models for the study of disorders of vascular blood vessels, such as arteriosclerosis as well as for the study of transplant arteriosclerosis.

A variety of methods are available for the production of transgenic animals associated with

this invention. DNA can be injected into the pronucleus of a fertilized egg before fusion of the male and female pronuclei, or injected into the nucleus of an embryonic cell (e.g., the nucleus of a two-cell embryo) following the initiation of cell division. See e.g., Brinster et al., *Proc. Nat. Acad. Sci. USA*, 82: 4438 (1985); Brinster et al., *cell* 27: 223 (1981); Costantini et al., *Nature* 294: 982 (1981); Harpers et al., *Nature* 293: 540 (1981); Wagner et al., *Proc. Nat. Acad. Sci. USA* 78:5016 (1981); Gordon et al., *Proc. Nat. Acad. Sci. USA* 73: 1260 (1976). The fertilized egg is then implanted into the uterus of the recipient female and allowed to develop into an animal.

An alternative method for producing transgenic animals involves the incorporation of the desired gene sequence into a virus which is capable of affecting the cells of a host animal. See e.g., Elbrecht et al., *Molec. Cell. Biol.* 7: 1276 (1987); Lacey et al., *Nature* 322: 609 (1986); Leopold et al., *Cell* 51: 885 (1987). Embryos can be infected with viruses, especially retroviruses, modified to carry the nucleotide sequences of the invention which encode FLIP proteins or sequences which disrupt the native FLIP gene to produce a knockout animal.

Another method for producing transgenic animals involves the injection of pluripotent embryonic stem cells into a blastocyst of a developing embryo. Pluripotent stem cells derived from the inner cell mass of the embryo and stabilized in culture can be manipulated in culture to incorporate nucleotide sequences of the invention. A transgenic animal can be produced from such cells through implantation into a blastocyst that is implanted into a foster mother and allowed to come to term. See e.g., Robertson et al., *Cold Spring Harbor Conference Cell Proliferation* 10: 647 (1983); Bradley et al., *Nature* 309: 255 (1984); Wagner et al., *Cold Spring Harbor Symposium Quantitative Biology* 50: 691 (1985).

The procedures for manipulation of the rodent embryo and for microinjection of DNA into the pronucleus of the zygote are well known to those of ordinary skill in the art (Hogan et al., supra). Microinjection procedures for fish, amphibian eggs and birds are detailed in Houdebine and Chourrout, *Experientia*, 47: 897-905 (1991). Other procedures for introduction of DNA into tissues of animals are described in U.S. Patent No., 4,945,050 (Sandford et al., July 30, 1990).

By way of example only, to prepare a transgenic mouse, female mice are induced to superovulate. Females are placed with males, and the mated females are sacrificed by CO<sub>2</sub> asphyxiation or cervical dislocation and embryos are recovered from excised oviducts. Surrounding cumulus cells are removed. Pronuclear embryos are then washed and stored until the time of injection. Randomly cycling adult female mice are paired with vasectomized males. Recipient females are mated at the same time as donor females. Embryos then are transferred



surgically. The procedure for generating transgenic rats is similar to that of mice. See Hammer et al., *Cell*, 63:1099-1112 (1990).

Methods for the culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection also are well known to those of ordinary skill in the art. See, for example, *Teratocarcinomas and Embryonic Stem Cells, A Practical Approach*, E.J. Robertson, ed., IRL Press (1987).

In cases involving random gene integration, a clone containing the sequence(s) of the invention is co-transfected with a gene encoding resistance. Alternatively, the gene encoding neomycin resistance is physically linked to the sequence(s) of the invention. Transfection and isolation of desired clones are carried out by any one of several methods well known to those of ordinary skill in the art (E.J. Robertson, *supra*).

DNA molecules introduced into ES cells can also be integrated into the chromosome through the process of homologous recombination. Capecchi, *Science*, 244: 1288-1292 (1989). Methods for positive selection of the recombination event (e.g., neo resistance) and dual positive-negative selection (e.g., neo resistance and gangcyclovir resistance) and the subsequent identification of the desired clones by PCR have been described by Capecchi, *supra* and Joyner et al., *Nature*, 338: 153-156 (1989). The final phase of the procedure is to inject targeted ES cells into blastocysts and to transfer the blastocysts into pseudopregnant females. The resulting chimeric animals are bred and the offspring are analyzed by Southern blotting to identify individuals that carry the transgene.

Procedures for the production of non-rodent mammals and other animals have been discussed by others. See Houdebine and Chourrout, *supra*; Pursel et al., *Science* 244: 1281-1288 (1989); and Simms et al., *Bio/Technology*, 6: 179-183 (1988).

Inactivation or replacement of the endogenous FLIP gene can be achieved by a homologous recombination system using embryonic stem cells. The resultant transgenic non-human mammals having a knockout FLIP characteristic may be used as a model for atherosclerosis. Vascular endothelial cells which are exposed to oxidized lipid may be predisposed to apoptosis and thus, result in vascular wall inflammation and produce an atherosclerotic phenotype. A variety of therapeutic drugs can be administered to the phenotypically atherosclerotic animals to determine the affect of the therapeutic drugs on vascular wall inflammation and/or endothelial cell apoptosis. In this manner, therapeutic drugs which are useful for inhibiting Fas ligand-mediated

apoptosis in vascular endothelial cells can be identified. Such agents are useful for, e.g., treating atherosclerosis.

Additionally, a normal or mutant version of FLIP can be inserted into the mouse germ line to produce transgenic animals which constitutively or inducible express the normal or mutant form of FLIP. These animals are useful in studies to define the role and function of FLIP in cells and, in particular, are useful for defining the role, function and efficacy of using fragments (alone or in combination) of the intact FLIP death effector and caspase-like domains.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

## **EXAMPLES**

### **Example 1.**

#### **Requirement for the FAS/FAS ligand pathway in vascular endothelial cell apoptosis induced by oxidized LDL**

It is firmly established that elevated plasma concentrations of low-density lipoprotein (LDL) are associated with accelerated atherogenesis (Goldstein and Brown, 1977; Group 1994; Steinberg, 1983), a prevalent disease in cultures with high fat diets. Recent evidence suggests that oxidative modification of LDL renders it more atherogenic (Parthasarathy *et al.*, 1992 Steinberg *et al.*, 1989; Witztum and Steinburg, 1991), and oxidized LDL (OxLDL) has been identified in atherosclerotic lesions (Haberland *et al.*, 1988; Ylä-Herttuala *et al.*, 1989). Sub-endothelial macrophages internalize OxLDL, contributing to lipid accumulation in the atheroma (Kodama, 1990). Endothelial cells, which form a protective lining on the luminal surface of the blood vessel wall, also internalize OxLDL via an endothelial cell-specific scavenger receptor (Sawamura *et al.*, 1997; VanBerkel *et al.*, 1991). OxLDL and its lipid constituents have numerous detrimental effects on endothelial cell function including the induction of apoptosis (Dimmeler *et al.* 1997; Escargueil-Blanc *et al.* 1997; Juckett *et al.*, 1995). However, the mechanism by which OxLDL induces endothelial cell apoptosis has not been delineated.

Recently, we demonstrated that vascular endothelial cells express FasL, which can function to induce apoptotic cell death in adherent leukocytes (Sata and Walsh, 1998). Under normal conditions, endothelial cells are resistant to Fas-mediated apoptosis although they express detectable Fas on their cell surface (Richardson *et al.*, 1994; Sata and Walsh, 1998 ). To

investigate whether OxLDL-induced endothelial cell apoptosis involves the Fas-FasL pathway, FACS analysis of cell surface FasL was performed on human umbilical vein endothelial cells (HUVECs) treated with OxLDL or lysophosphatidylcholine (LPC), a component of OxLDL. Incubation with OxLDL or LPC markedly upregulated FasL expression on HUVECs (Fig. 1) and on human aortic endothelial cells (HAECs). In contrast, Fas expression was not affected by treatment with OxLDL or LPC.

HUVECs treated with OxLDL displayed characteristics of apoptosis including cell shrinkage and nuclear condensation, DNA fragmentation, and decreased mitochondrial function (Fig. 2). Apoptosis was markedly attenuated when cultures were co-incubated with a neutralizing anti-FasL antibody, demonstrating that FasL is essential for the induction of endothelial cell apoptosis by OxLDL. Similar observations were also made with HAECs (not shown). Interestingly, OxLDL-treated endothelial cells protected from death attained a distinctive elongated cell morphology, indicating that other actions of OxLDL are not blocked by the neutralizing anti-FasL antibody.

Consistent with previous observations (Richardson *et al*, 1994; Sata and Walsh, 1998), untreated HUVECs and HAECs were resistant to Fas mediated apoptosis, as incubation with an agonistic anti-Fas antibody did not result in DNA fragmentation or loss in mitochondrial function (Fig. 2A, 2B). However, apoptosis could be induced in the presence of OxLDL by exposure to the agonistic anti-Fas antibody when endogenous FasL was blocked by the neutralizing antibody. These data indicate that treatment with OxLDL sensitizes endothelial cells to Fas-mediated apoptosis, perhaps by bringing about changes in the intracellular Fas-signaling pathway.

Cells expressing both FasL and Fas can become dramatically sensitized to the Fas-mediated apoptosis in response to specific stimuli. It is well established that T lymphocyte number is controlled by a delayed sensitization to Fas-mediated apoptosis following activation (Klas, 1993). Perturbations in the Fas/FasL cell suicide pathway may also be important in determining the viability of transformed cells (Hueber *et al.*, 1997; Muller *et al.*, 1997), and there is increasing evidence that cancer cell sensitivity to Fas-mediated apoptosis is a key feature of tumor progression (Hahne *et al.*, 1996; O'Connell *et al.*, 1996; Strand *et al.*, 1996). Here, we presented evidence that alterations in sensitivity to Fas-mediated cell suicide may also have a role in determining the viability of endothelial cells to injurious agents. As injuries to endothelium trigger inflammatory-fibroproliferative processes in the vessel wall (Ross, 1993), the ability of OxLDL to sensitize ECs to FasL/Fas-mediated suicide may contribute to the accelerated

atherosclerosis seen in patients with hyperlipemia. Our data also suggest that alterations in Fas-mediated cell suicide may have a more widespread role in disease processes than was previously appreciated.

#### 5     **Example 1: Materials and Methods:**

Methods: HUVECs were cultured in EGM medium (Clonetics). LDL was isolated by sequential ultracentrifugation ( $d=1.019-1.063$ ) from freshly drawn, citrated normolipidemic human plasma to which EDTA was added. LDL was oxidized in the presence of  $\text{CuSO}_4$  for 24 hours at  $25^\circ\text{C}$  and the degree of oxidation was assessed by the increase of mobility on 1% agarose gel (1.4 versus native LDL)(Galle *et al.*, 1995). 90% confluent HUVECs were incubated with  
10     150  $\mu\text{g}$  protein/ml of OxLDL or 45  $\mu\text{M}$  of  $\text{L-}\alpha$ -palmitoyl lysophosphatidyl choline for 13 hours. HUVECs were detached from the culture plate with 0.5% EDTA and incubated with an anti-FasL polyclonal antibody (C-20, Santa Cruz) (filled curve) or with a rabbit IgG (open curve) in PBS with 10% FBS, followed by incubation with an FITC-conjugated anti-rabbit Ig antibody.  
15     Immunofluorescence staining was analyzed by FACS (fluorescence activated cell sorter) (Becton Dickinson).

To determine whether OxLDL induces apoptosis and morphological changes, HAECs (~90% confluent) were incubated with or without OxLDL (300  $\mu\text{g}$  protein/ml) in the absence and presence of an anti-FasL antibody (10  $\mu\text{g}/\text{ml}$ , 4H9, MBL, Nagoya) that is capable of neutralizing  
20     FasL (Tanaka *et al.*, 1996). After 22 hours of incubation, cells were fixed in 4% paraformaldehyde, stained with Hoechst 33258 (Sigma), and observed under the microscope equipped with a phase-contrast and epifluorescence optics (x40) lens.

#### **Example 1 Reference List:**

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**Example 2.****Construction of a replication defective adenovirus encoding FLIP.**

Endothelial cells express Fas receptor but are not normally susceptible to Fas ligand-mediated cell death; however, dysfunctional endothelial cells are susceptible to Fas ligand-mediated apoptosis. To demonstrate that a normal physiological role of FLIP is to protect endothelial cells from Fas ligand-mediated apoptosis, an Adeno-FLIP construct (Fig. 7) is prepared as described below and utilized to characterize the differential effects of FLIP expression on endothelial cell viability in vitro (in the presence or absence of Fas ligand, with or without oxidized lipid). This construct also is used to assess the effects of increased apoptosis on lesion formation in the rat carotid and rabbit iliac models of vascular injury. It is believed that the endothelial cells that are engineered to express FLIP behave as normal endothelial cells even in the presence of oxidized lipid and, in this manner, dysfunctional endothelial cells (susceptible to Fas ligand-mediated apoptosis) can be converted into normal, Fas ligand-resistant cells.

To understand the role of apoptosis in vessel wall lesion formation and remodeling, a replication defective adenovirus encoding the FLIP in models of vascular injury is used. Different preparations of this adenovirus (designated "Adeno-FLIP") are described above.

Briefly, a *constitutively expressed* Adeno-FLIP construct is made by subcloning a FLIP cDNA (based upon human FLIP-L or FLIP-S sequences as shown in SEQ. ID Nos. 1 or 3), downstream from an appropriate expression cassette (for example, the CMV promoter/enhancer) into the EcoRV site of the pCO1 vector containing the Ad5 adenoviral sequences required for homologous recombination.

The resulting plasmids are linearized by restriction enzyme digestion and cotransfected in 293 cells with large ClaI fragment of the Ad5 d1324 viral DNA (Stratford-Perricaudet, L.D., et al., 1993, *J. Clin. Invest.* 90:626-630). The resulting replication-defective recombinant adenoviral constructs are purified from isolated plaques. The viral preparations are purified by two CsCl gradient centrifugations, dialyzed against buffer containing 10 mM Tris-Cl pH 7.5, 1 mM MgCl<sub>2</sub>, and 135 mM NaCl and stored at -80°C in 10% glycerol. Viral titer is determined by plaque assay on 293 cells (Graham, F.L., and A.J. van der Eb, 1973, *Virology* 52:456-463) and expressed as plaque forming units (pfu) per ml.

Alternatively, an *inducibly expressed* Adeno-FLIP construct is made by subcloning a FLIP cDNA (based upon human FLIP-L or FLIP-S sequences as shown in SEQ. ID Nos. 1 or 3), downstream from the tetracycline-responsive element (TRE) of the Clontech, (Palo Alto,

CA)Tet-Off™ & Tet-On™ Gene Expression System according to manufacturer's instructions and standard molecular biological techniques. In certain embodiments, a readily detectible epitope may also be incorporated in the construct (for example, a FLAG sequence epitope placed 5' to the FLIP cDNA). This cassette, can then be placed into a pdE1Sp1A adenovirus vector (Microbix, Ontario, CANADA) (e.g. at XhoI-HindIII sites of pdE1Sp1A). Similarly, the PhCMV-rtTA containing cassette required for the inducible system can also be placed into a pdE1Sp1A adenovirus vector. The resulting two constructs may then be linearized and transfected to produce replication-defective recombinant adenoviral constructs. Once the two recombinant adenoviral constructs are purified from isolated plaques, they can be co-transfected to produce a tetracycline-inducible system in vivo and in vitro. Examples of similar (*nonFLIP*) inducible adenoviral constructs are also described in Harding TC, et al., 1998, *Nat Biotechnol.*, 16(6):553-5, and Harding TC, et al., 1997, *J Neurochem.*, 69(6):2620-3.

The delivery of Adeno-FLIP constructs at the site of vascular injury create a local region of endothelial cells that are protected from Fas ligand-mediated apoptotic cell death.

#### Example 2 Materials and Methods:

An overview of the materials and methods for several of the procedures for preparing a replication-defective recombinant adenoviral vector containing the cDNA encoding FLIP and delivering the recombinant viral vector by percutaneous arterial gene transfer are presented below.

**Recombinant adenoviral vectors.** Replication-defective recombinant adenoviral vectors, based on human adenovirus 5 serotype, are produced as previously described (Stratford-Perricaudet, L.D., et al., 1993, *J. Clin. Invest.* 90:626-630). The human FLIP gene cDNA (based upon SEQ. ID Nos. 1 or 3) is inserted between the XbaI and BamHI sites of the pCG vector (Tanaka, M., and W. Herr, 1990, *Cell* 60:375-386) resulting in the placement of the FLIP gene downstream from the cytomegalovirus (CMV) early promoter and the herpes simplex virus and thymidine kinase gene 5' untranslated region (UTR). The XmnI-SfiI fragment from pCG-FLIP is then inserted at the EcoRV site of the pCO1 vector containing the Ad5 adenoviral sequence required for homologous recombination. The resulting plasmid is linearized by XmnI and cotransfected in 293 cells with the large fragment of the Ad5 d1324 viral DNA (Stratford-Perricaudet, L.D., et al., 1993, *J. Clin. Invest.* 90:626-630). The resulting replication-defective recombinant adenoviruses are purified from isolated plaques and viral DNA prepared.

Recombinant adenoviruses containing the FLIP cDNA are identified by restriction fragment analysis and amplified in 293 cells. The viral preparations used for both *in vivo* studies are purified by 2 CsCl gradient centrifugations, dialysed against buffer containing 10 mM Tris-Cl pH 7.5, 1 mM MgCl<sub>2</sub>, and 135 mM NaCl and stored at -80°C in 10% glycerol. Viral titer is  
5 determined by plaque assay on 293 cells as previously described (Graham, FL, and AJ van der Eb, 1973, Virology 52:456-463) and expressed as plaque forming units 5 (pfu) per ml. The construction of the control Ad-βgal used in this work has been previously described (Stratford-Perricaudet, L.D., et al., 1993, *J. Clin. Invest.* 90:626-630).

Assays to assess percutaneous arterial gene transfer and balloon angioplasty *in vivo* are  
10 performed as previously described.

It should be understood that the preceding is merely a detailed description of certain preferred embodiments. It therefore should be apparent to those skilled in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention. It is intended to encompass all such modifications within the scope of the appended  
15 claims.

All references, patents and patent publications that are recited in this application are incorporated in their entirety herein by reference.

What is claimed is presented below and is followed by an Abstract and a Sequence Listing.

I claim:



CLAIMS

1. A method for treating a condition characterized by vascular wall inflammation in a subject comprising:

5 administering a FLIP molecule to a subject in need of such treatment in an amount effective to inhibit Fas ligand-mediated apoptosis of vascular endothelial cells in the subject.

2. The method of claim 1, wherein the condition is further characterized by elevated vascular levels of oxidized lipid.

10 3. The method of claim 1, wherein the FLIP molecule is a FLIP nucleic acid.

4. The method of claim 3, wherein the FLIP nucleic acid is a nucleic acid encoding a FLIP polypeptide selected from the group consisting of:

15 (a) an intact FLIP polypeptide;

(b) a fragment of a FLIP polypeptide comprising one or more FLIP death effector domains;

(c) a fragment of a FLIP polypeptide comprising an amino terminus containing one or more FLIP death effector domains selected from DED I and DED II and a carboxy terminus containing one or more FLIP caspase-like subunits; and

20 (d) a fragment of a FLIP polypeptide comprising one or more FLIP caspase-like subunits.

5. The method of claim 4, wherein the intact FLIP polypeptide has an amino acid sequence of SEQ. ID NO.2.

25 6. The method of claim 1, wherein the FLIP molecule is a FLIP polypeptide.

7. The method of claim 6, wherein the FLIP polypeptide is selected from the group consisting of:

(a) an intact FLIP polypeptide;

30 (b) a fragment of a FLIP polypeptide comprising one or more FLIP death effector domains;

(c) a fragment of a FLIP polypeptide comprising an amino terminus containing one or more FLIP death effector domains and a carboxy terminus containing one or more FLIP caspase-

like subunits; and

(d) a fragment of a FLIP polypeptide comprising one or more FLIP caspase-like subunits.

5 8. The method of claim 7, wherein the intact FLIP polypeptide has an amino acid sequence of SEQ. ID NO.2.

9. The method of claim 1, wherein the condition is selected from the group consisting of:  
(a) atherosclerosis;  
(b) transplant arteriosclerosis; and  
10 (c) vascular injury.

10. A method for treating a subject who is or will be a transplant organ recipient comprising:  
administering a FLIP molecule to a subject in need of such treatment in an amount effective  
to inhibit the development of atherosclerotic legions within the vessels of the transplanted organ,  
15 wherein the subject is otherwise free of symptoms calling for FLIP molecule treatment.

11. The method of claim 10, wherein the transplanted organ is a heart or a kidney.

12. The method of claim 10, wherein the FLIP molecule is administered to a subject with an  
20 arterial occlusion in conjunction with treatment of said occlusion.

13. A method for inhibiting the development or progression of arteriosclerotic legions in a  
subject otherwise free of symptoms calling for FLIP treatment, comprising:  
administering a FLIP molecule to a subject in need of such treatment in an amount effective  
25 to inhibit the development or progression of arteriosclerotic legions, wherein the subject is  
otherwise free of symptoms calling for treatment with a FLIP molecule.

14. An isolated nucleic acid molecule  
(a) which is or hybridizes under stringent conditions to a molecule consisting of the nucleic  
30 acid sequence of SEQ. ID NO. 1 and which codes for FRAGMENT FLIP polypeptide,  
(b) nucleic acid molecules that differ from the nucleic acid molecules of (a) in codon  
sequence due to the degeneracy of the genetic code, and

(c) complements of (a) and (b).

15. The isolated nucleic acid molecule of claim 14, wherein the isolated nucleic acid molecule encodes a polypeptide selected from the group consisting of:

- 5 (a) a fragment of a FLIP polypeptide comprising one or more FLIP death effector domains;
- (b) a fragment of a FLIP polypeptide comprising an amino terminus containing one or more FLIP death effector domains and a carboxy terminus containing one or more FLIP caspase-like subunits; and
- (c) a fragment of a FLIP polypeptide comprising one or more FLIP caspase-like subunits.

10 16. A vector comprising the isolated nucleic acid molecule of claim 14, operably linked to a promoter.

17. A host cell transformed or transfected with an expression vector comprising the isolated  
15 nucleic acid molecule of claim 14, operably linked to a promoter.

18. An isolated polypeptide coded for by the isolated nucleic acid molecule of claim 14.

19. An isolated nucleic acid molecule

- 20 (a) which hybridizes under stringent conditions to a molecule consisting of the nucleic acid sequence of SEQ. ID NO. 1 and which codes for an intact FLIP polypeptide, wherein the intact FLIP polypeptide is capable of autoproteolysis;
- (b) nucleic acid molecules that differ from the nucleic acid molecules of (a) in codon sequence due to the degeneracy of the genetic code, and
- 25 (c) complements of (a) and (b).

20. The isolated nucleic acid molecule of claim 19, wherein the isolated nucleic acid molecule consists essentially of SEQ. ID NO. 1, provided that the codons of SEQ. ID. NO. 1 that encode the caspase-like domain cleavage site encode a FLIP polypeptide that is capable of  
30 autoproteolysis.

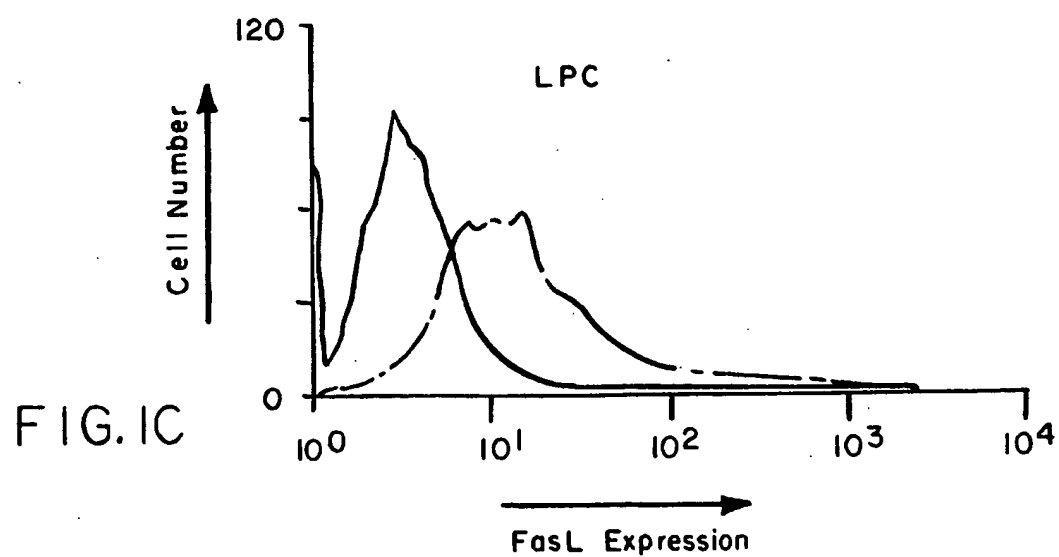
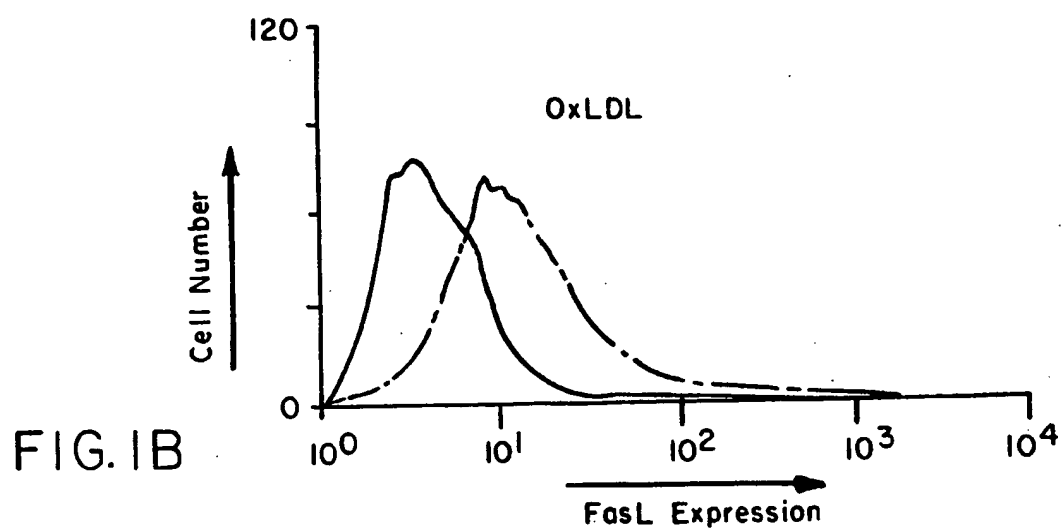
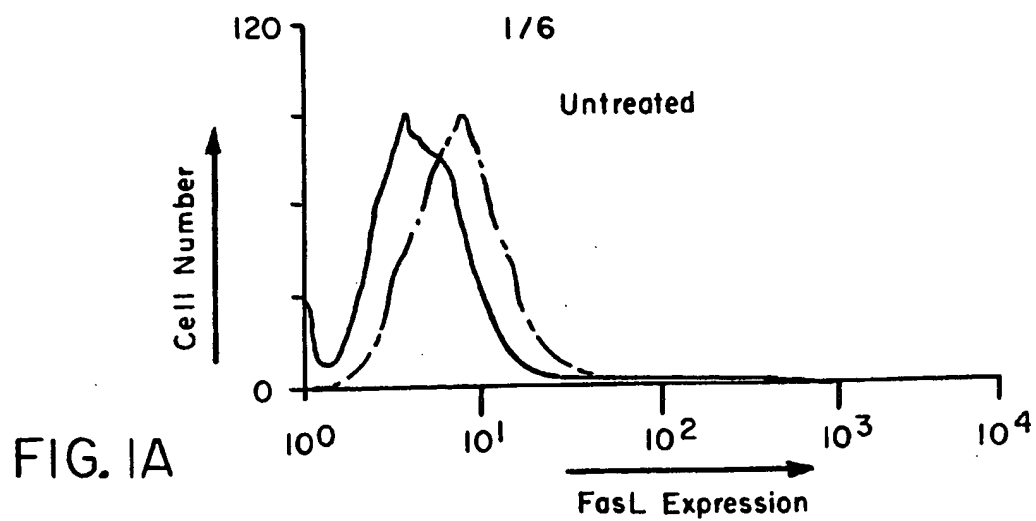
21. A vector comprising the isolated nucleic acid molecule of claim 19, operably linked to a promoter.
22. A host cell transformed or transfected with an expression vector comprising the isolated  
5 nucleic acid molecule of claim 19, operably linked to a promoter.
23. An isolated polypeptide coded for by the isolated nucleic acid molecule of claim 19.
24. A screening method for selecting an inhibitory agent that inhibits the development or  
10 progression of atherosclerotic lesions, the method comprising,  
(1) contacting a cell that expresses FLIP with an oxidized lipid and a putative inhibitor  
under conditions wherein the cell is capable of undergoing Fas ligand-induced apoptosis;  
(2) determining whether the cell undergoes Fas ligand-induced apoptosis in the presence  
of the putative inhibitor; and  
15 (3) selecting the putative inhibitor that inhibits or prevents Fas ligand-induced apoptosis  
as an agent that inhibits the development or progression of atherosclerotic lesions.
25. The method of claim 24, wherein the conditions include adding a Fas ligand or a Fas  
ligand mimic such as an antibody that binds to a Fas receptor and induces apoptosis.  
20
26. The method of claim 24, wherein the cell is a vascular endothelial cell.
27. The method of claim 24, wherein the oxidized lipid is oxidized LDL.
28. The method of claim 24, wherein the putative inhibitor is contained in a combinatorial  
25 library.
29. A kit comprising one or more of the following, alone or in combination:  
(1) an oxidized lipid;  
30 (2) a cell that expresses FLIP and that is capable of undergoing Fas ligand-induced

apoptosis when FLIP expression is down regulated;

(3) a Fas ligand or a Fas ligand mimic such as an antibody that binds to a Fas receptor and induces apoptosis; and

5 (4) instructions for inducing apoptosis and determining whether a test compound inhibits or prevents Fas ligand-induced apoptosis.

30. A method for inhibiting Fas ligand-mediated apoptosis in an endothelial cell comprising:  
contacting an endothelial cell with an isolated FLIP molecule under conditions to introduce  
the FLIP molecule into the endothelial cell, wherein the FLIP molecule is present in  
10 an amount sufficient to inhibit apoptosis that is induced by contacting the cell with an oxidized  
lipid.



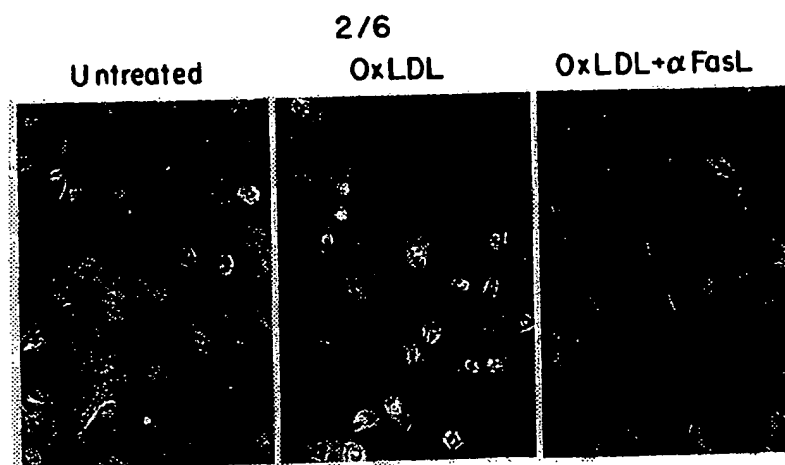


FIG. 2A

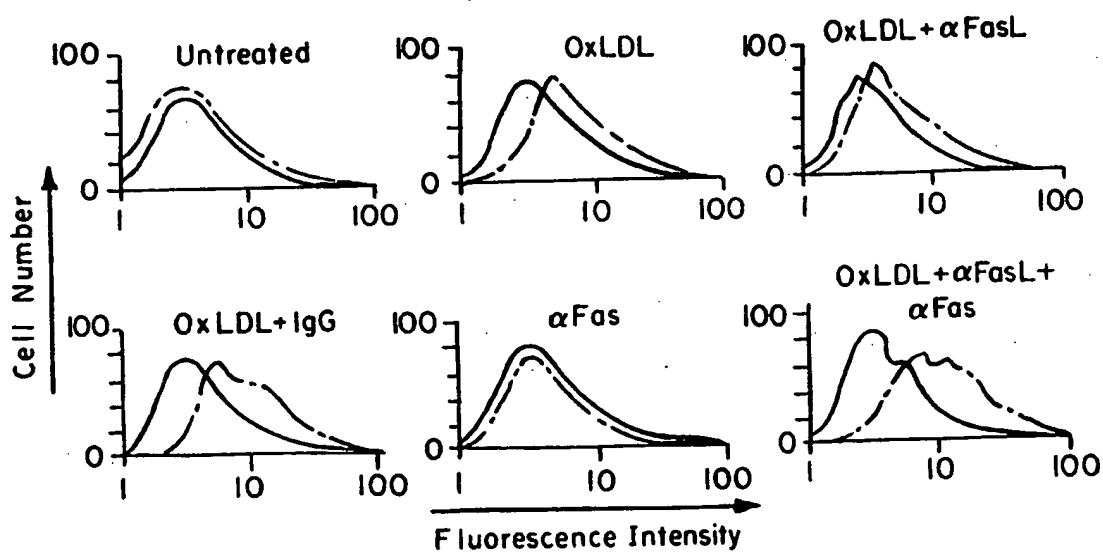


FIG. 2B

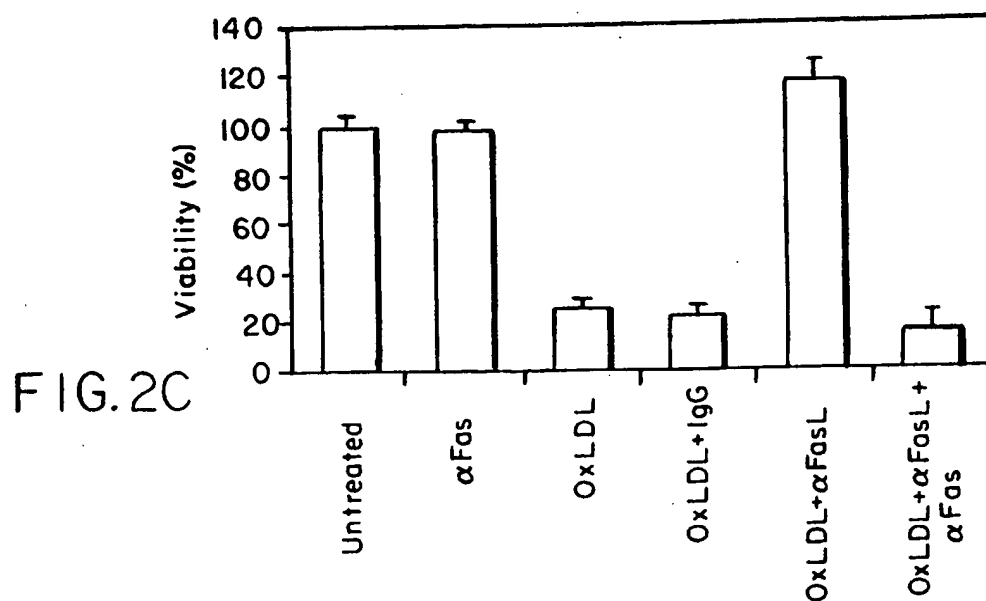


FIG. 2C

FIG.3A

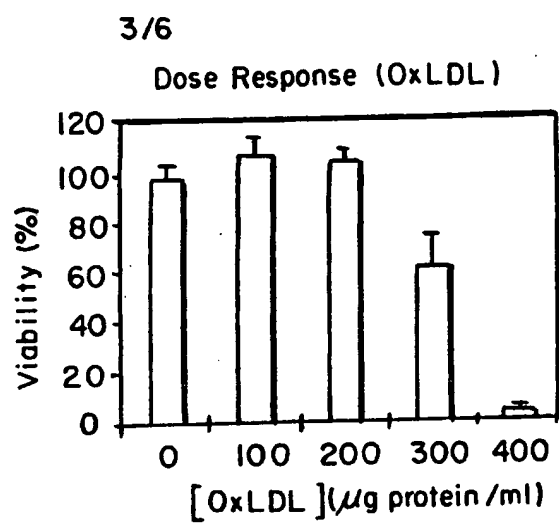


FIG.3B

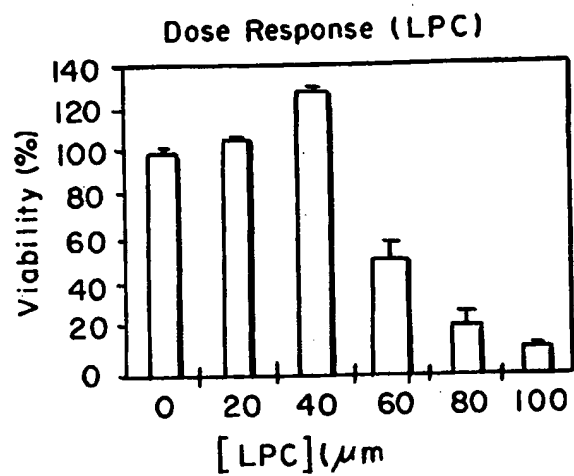
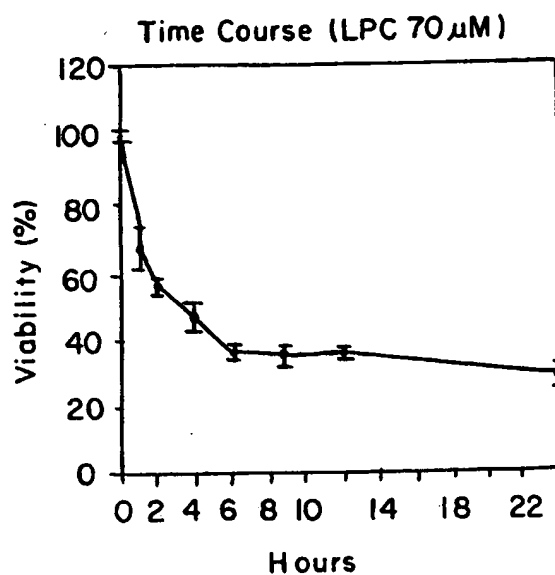


FIG.3C





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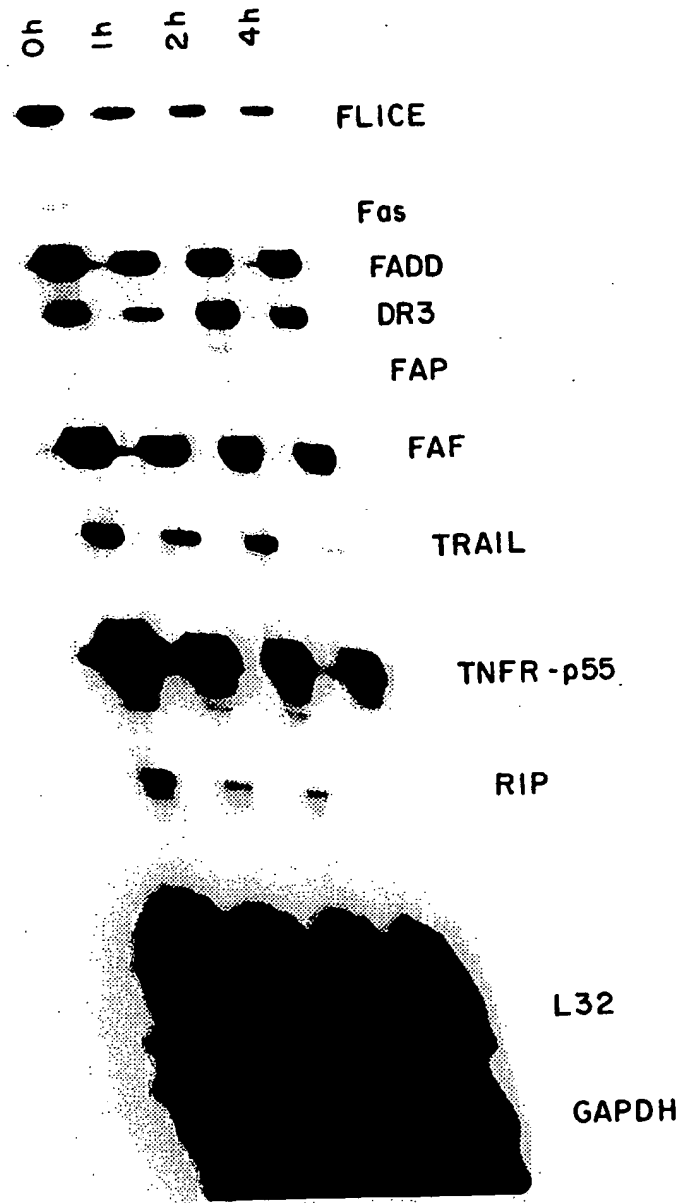


FIG. 4

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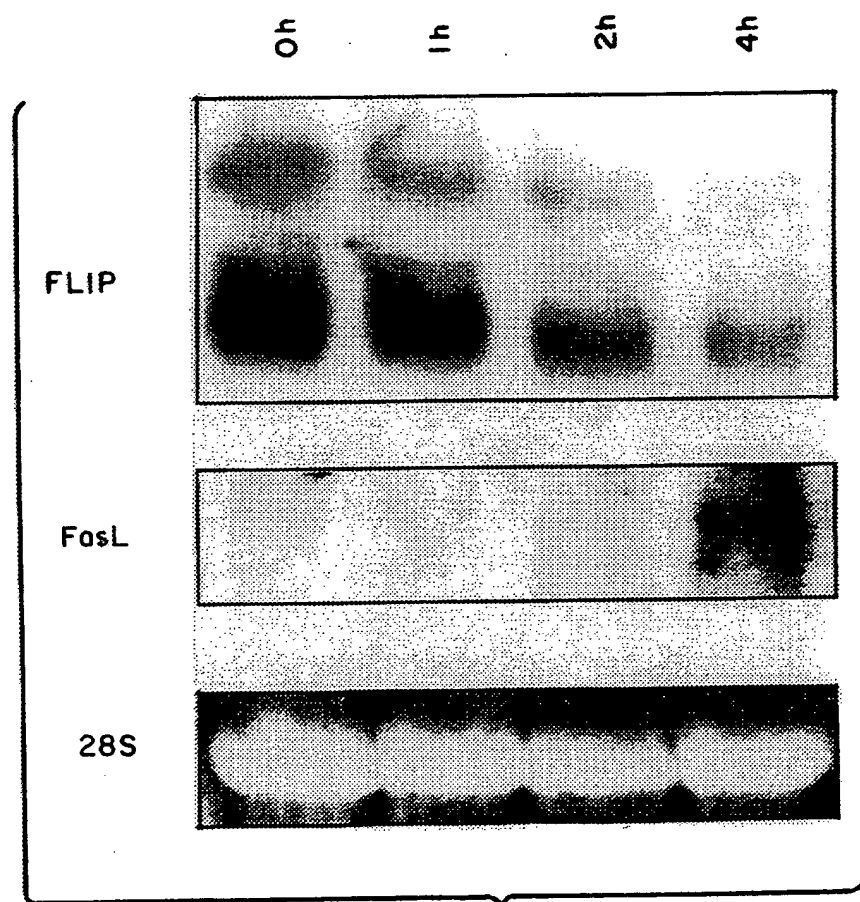


FIG.5

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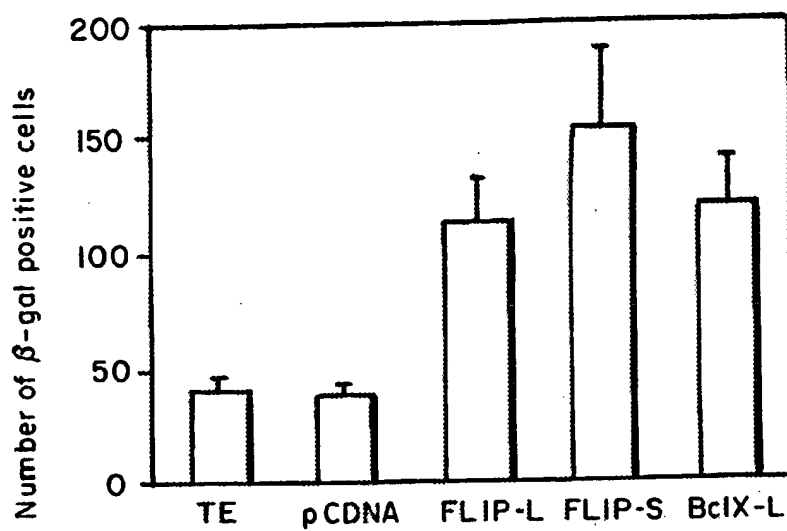


FIG. 6

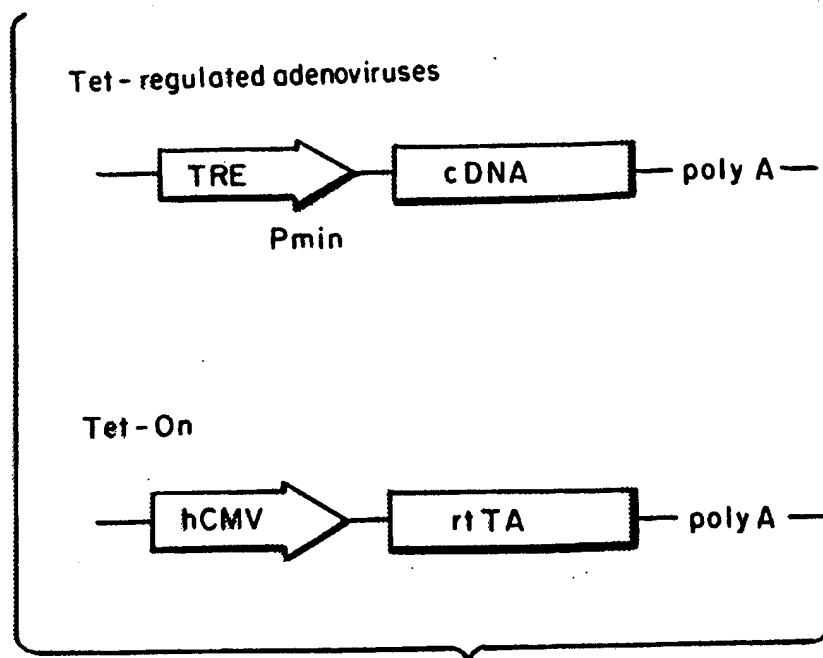


FIG. 7

## SEQUENCE LISTING

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Walsh, Kenneth

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Lys	Met	Phe	Phe	Ile	Gln	Asn	Tyr	Val	Val	Ser	Glu	Gly	Gln	Leu	Glu
		355				360				365					
Asp	Ser	Ser	Leu	Leu	Glu	Val	Asp	Gly	Pro	Ala	Met	Lys	Asn	Val	Glu

370                      375                      380  
 Phe Lys Ala Gln Lys Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp  
 385                      390                      395                      400  
 Phe Phe Trp Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser  
                     405                      410                      415  
 His Ser Ser Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg  
                     420                      425                      430  
 Gln Glu Arg Lys Arg Pro Leu Leu Asp Leu His Ile Glu Leu Asn Gly  
                     435                      440                      445  
 Tyr Met Tyr Asp Trp Asn Ser Arg Val Ser Ala Lys Glu Lys Tyr Tyr  
                     450                      455                      460  
 Val Trp Leu Gln His Thr Leu Arg Lys Lys Leu Ile Leu Ser Tyr Thr  
 465                      470                      475                      480

&lt;210&gt; 3

&lt;211&gt; 1062

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 3

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gcacgagcgg cacgagtaga cttctataga tccctttcta tagaacttaa tctacttaag      60
tcagggagac caccagaag gaaagagccc ataactttcaa tcttaggcatt aagttagctt      120
gataagattt tcagaaaaat tcccttttaa ccacagaact cccccactgg aaaggattct      180
gaaagaaatg aagtcagccc tcagaaatga agttgactgc ctgctggcct tctgttgact      240
ggcccggagc tgtactgcaa gacccttggt agcttcctta gtctaagagt aggatgtctg      300
ctgaagtcatt ccatcaggtt gaagaagcac ttgatacaga tgagaaggag atgctgctct      360
ttttgtgccg ggatgttgct atagatgtgg ttccacctaa tgtcagggac cttctggata      420
ttttacggga aagaggttaag ctgtctgtcg gggacttggc tgaactgctc tacagagtga      480
ggcgatttga cctgctcaaa cgtatcttga agatggacag aaaagctgtg gagaccacc      540
tgctcaggaa cctcacctt gtttcggact atagagtgtc gatggcagag attggtgagg      600
atgttgataa atctgatgtg tcctcattaa ttttcctcat gaaggattac atgggccgag      660
gcaagataag caaggagaag agtttcttgg accttggtgt tgagttggag aaactaaatc      720
tggttgcccc agatcaactg gatttattag aaaaatgcct aaagaacatc cacagaatag      780
acctgaagac aaaaatccag aagtacaagc agtctgttca aggagcaggg acaagttaca      840
ggaatgttct ccaagcagca atccaaaaga gtctcaagga tccttcaaata aacttcagga      900
tgataacacc ctatgcccatt tgcctgatac tgaaaattct tggaaattgt tccatgtgat      960
taacatggaa ctgcctctac ttaatcattc tgaatgatta aatcgtttca ttttctaaat     1020
gtgtaaaaaa aaaaaaaaaa aaaaaaactc gagggggggc cc                        1062
  
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&lt;210&gt; 4

&lt;211&gt; 221

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 4

```

Met Ser Ala Glu Val Ile His Gln Val Glu Glu Ala Leu Asp Thr Asp
  1             5             10             15
Glu Lys Glu Met Leu Leu Phe Leu Cys Arg Asp Val Ala Ile Asp Val
          20             25             30
Val Pro Pro Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly
        35             40             45
Lys Leu Ser Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg
        50             55             60
Phe Asp Leu Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu
65             70             75             80
Thr His Leu Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu
          85             90             95
Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu
        100            105            110
Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu
        115            120            125
Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val
        130            135            140
Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His
145            150            155            160
Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln
          165            170            175
Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys
        180            185            190
Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Met Ile Thr Pro Tyr Ala
        195            200            205
His Cys Pro Asp Leu Lys Ile Leu Gly Asn Cys Ser Met
        210            215            220

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&lt;210&gt; 5

&lt;211&gt; 2413

&lt;212&gt; DNA



&lt;213&gt; Mus Musculus

&lt;400&gt; 5

gaattccgag cctctcaagc ggccacttag ggccggacag agtgtctcta ttgcaagaac	60
tctgagagaa atgaagagag tcctcagcaa tgatgttggc ttctcgtggt tcccagagcc	120
ctgcttaatg gatggagact ggacgagaac ctggctgctg tggttctgaa catggcccag	180
agccctgtgt ctgccgaggt cattcaccag gtggaagagt gtcttgatga agacgagaag	240
gagatgatgc tcttcctgtg tagagatgtg actgagaacc tggctgcacc taacgtcagg	300
gacctcctgg atagcttaag tgagagaggc cagctctctt ttgctacctt ggctgaattg	360
ctctacagag tgaggcgggt tgaccttctc aagaggatct tgaagacaga caaagcaacc	420
gtggaggacc acctgcgcag aaaccctcac ctggtttctg attatagggc cctgctgatg	480
gagattggtg agagcttaga tcagaacgat gtatcctcct tagttttcct tacaagggat	540
tacacaggca gaggcaagat agccaaggac aagagtttct tggatctggg gattgaattg	600
gagaaactga atctaattgc ttcagaccaa ttgaatttgt tagaaaaatg cctgaagaac	660
atccacagaa tagacttgaa cacaaagatc cagaagtaca ccagtcacag ccaaggagca	720
agatcaaata tgaatactct ccaggcttcg ctcccaaaat tgagtatcaa gtataactca	780
aggctccaga atgggcgaag taaagagcca agatttgtgg aataccgtga cagtcaaaga	840
acactgggtg agacatccat ccaggaatca ggagcttttt tacctccgca catccgtgaa	900
gagacttaca ggatgcagag caagccccta ggaatctgct tgatcattga ttgtattggc	960
aacgacacaa aatatcttca agagaccttc acttccctgg gctatcatat ccagcttttc	1020
ttgtttccca agtcacatga cataaccagc attgttcgcc gatatgcaag tatggcccaa	1080
catcaagact atgacagctt tgcattgtgt ctggtgagcc tagggaggct ccaaagcatg	1140
atgggcagag atcaagttca ctccagggttc tccttgatc atgtcaagaa catgttcacg	1200
ggggacacgt gcccttctct cagagggaag ccaaagctct tttttattca gaactatgag	1260
tcgttaggta gccagttgga agatagcagc ctggaggtag atggggccatc aataaaaaat	1320
gtggactcta agcccctgca acccagacac tgcacaactc acccagaagc tgatatcttt	1380
tggagcctgt gcacagcaga cgtatctcac ttggagaagc cctccagctc atcctctgtg	1440
tatctgcaga agctctccca gcagctgaag caaggcagga gacgcccact cgtggacctc	1500
cacgttgaaac tcatggacaa agtgtatgag tggaaacagt gtgtttcgtc taaggagaaa	1560
tacagcctca gcctgcagca cactctgagg aagaaactca tcctggctcc tacgtgagaa	1620
ccccagaccg ttggtgttct tggatatca tccagggtgg cggcttgag cagagcttgg	1680
cggttacggc tgcttctggc tgcttctggc tctgccgtga gtccctggcct agggttctcc	1740
tgtgcacagg catgagccgt aacctgtgct ctgggaaacg tctcactccc gccgccgtgc	1800
ctttacctct ctaaacttcc ctacttacat tccttagtgc gatgttttgc cagagtgtgg	1860
agaacagtaa gacataaacc tattgtttgt ttgttttttt gggggggagg ttatctacca	1920
agttatacca agttattgta tgggtgtata gtgtatagt gttcaagatt tgacactgaa	1980
tgtaacttga gacttacctg agtttgcct gcgactgggt aaattgttct tatggcacat	2040
ctaatacttt aataagtaat tacctcatta agtaccatt gcttcaggac tttcacattg	2100

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gccaccaatt tctgtgaccc agctccacat ttatattctc tttctgcaaa accaaatttc      2160
attatgtctg tttaatatct acagtctaag gccttgtaag acatctagat agaaaaatag      2220
ttacccatga gcacagaagg gctggcctga ccttcaccag ctgtgcagtg gcttcggtga      2280
aggagaatga gccctactcc ttgaagggtg tagtgcttgg gagagcagtc tgtaccttgc      2340
ctgggcagca cagtagagcc agccccaaga acacaacagt gagtggggga gcttgccttg      2400
gttggtcag gat                                                                2413

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&lt;210&gt; 6

&lt;211&gt; 481

&lt;212&gt; PRT

&lt;213&gt; Mus Musculus

&lt;400&gt; 6

```

Met Ala Gln Ser Pro Val Ser Ala Glu Val Ile His Gln Val Glu Glu
 1             5             10             15
Cys Leu Asp Glu Asp Glu Lys Glu Met Met Leu Phe Leu Cys Arg Asp
          20             25             30
Val Thr Glu Asn Leu Ala Ala Pro Asn Val Arg Asp Leu Leu Asp Ser
          35             40             45
Leu Ser Glu Arg Gly Gln Leu Ser Phe Ala Thr Leu Ala Glu Leu Leu
          50             55             60
Tyr Arg Val Arg Arg Phe Asp Leu Leu Lys Arg Ile Leu Lys Thr Asp
          65             70             75             80
Lys Ala Thr Val Glu Asp His Leu Arg Arg Asn Pro His Leu Val Ser
          85             90             95
Asp Tyr Arg Val Leu Leu Met Glu Ile Gly Glu Ser Leu Asp Gln Asn
          100            105            110
Asp Val Ser Ser Leu Val Phe Leu Thr Arg Asp Tyr Thr Gly Arg Gly
          115            120            125
Lys Ile Ala Lys Asp Lys Ser Phe Leu Asp Leu Val Ile Glu Leu Glu
          130            135            140
Lys Leu Asn Leu Ile Ala Ser Asp Gln Leu Asn Leu Leu Glu Lys Cys
          145            150            155            160
Leu Lys Asn Ile His Arg Ile Asp Leu Asn Thr Lys Ile Gln Lys Tyr
          165            170            175
Thr Gln Ser Ser Gln Gly Ala Arg Ser Asn Met Asn Thr Leu Gln Ala
          180            185            190
Ser Leu Pro Lys Leu Ser Ile Lys Tyr Asn Ser Arg Leu Gln Asn Gly

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195	200	205
Arg Ser Lys Glu Pro Arg Phe Val Glu Tyr Arg Asp Ser Gln Arg Thr		
210	215	220
Leu Val Lys Thr Ser Ile Gln Glu Ser Gly Ala Phe Leu Pro Pro His		
225	230	235
Ile Arg Glu Glu Thr Tyr Arg Met Gln Ser Lys Pro Leu Gly Ile Cys		
245	250	255
Leu Ile Ile Asp Cys Ile Gly Asn Asp Thr Lys Tyr Leu Gln Glu Thr		
260	265	270
Phe Thr Ser Leu Gly Tyr His Ile Gln Leu Phe Leu Phe Pro Lys Ser		
275	280	285
His Asp Ile Thr Gln Ile Val Arg Arg Tyr Ala Ser Met Ala Gln His		
290	295	300
Gln Asp Tyr Asp Ser Phe Ala Cys Val Leu Val Ser Leu Gly Gly Ser		
305	310	315
Gln Ser Met Met Gly Arg Asp Gln Val His Ser Gly Phe Ser Leu Asp		
325	330	335
His Val Lys Asn Met Phe Thr Gly Asp Thr Cys Pro Ser Leu Arg Gly		
340	345	350
Lys Pro Lys Leu Phe Phe Ile Gln Asn Tyr Glu Ser Leu Gly Ser Gln		
355	360	365
Leu Glu Asp Ser Ser Leu Glu Val Asp Gly Pro Ser Ile Lys Asn Val		
370	375	380
Asp Ser Lys Pro Leu Gln Pro Arg His Cys Thr Thr His Pro Glu Ala		
385	390	395
Asp Ile Phe Trp Ser Leu Cys Thr Ala Asp Val Ser His Leu Glu Lys		
405	410	415
Pro Ser Ser Ser Ser Val Tyr Leu Gln Lys Leu Ser Gln Gln Leu		
420	425	430
Lys Gln Gly Arg Arg Arg Pro Leu Val Asp Leu His Val Glu Leu Met		
435	440	445
Asp Lys Val Tyr Ala Trp Asn Ser Gly Val Ser Ser Lys Glu Lys Tyr		
450	455	460
Ser Leu Ser Leu Gln His Thr Leu Arg Lys Lys Leu Ile Leu Ala Pro		
465	470	475
Thr		480

&lt;210&gt; 7

&lt;211&gt; 932

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 7

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atgtctgctg aagtcaccca tcaggttgaa gaagcacttg atacagatga gaaggagatg      60
ctgctctttt tgtgccgga tgttgctata gatgtggttc cacctaattgt cagggacctt      120
ctggatattt tacgggaaag aggtaagctg tctgtcgggg acttggtgga actgctctac      180
agagtgaggg gatttgacct gctcaaactg atcttgaaga tggacagaaa agctgtggag      240
acccacctgc tcaggaaccc tcaccttggt tcggactata gagtgtgat ggcagagatt      300
ggtgaggatt tggataaatc tgatgtgtcc tcattaattt tcctcatgaa ggattacatg      360
ggccgaggca agataagcaa ggagaagagt ttcttgacc ttgtggttga gttggagaaa      420
ctaaatctgg ttgccccaga tcaactggat ttattagaaa aatgcctaaa gaacatccac      480
agaatagacc tgaagacaaa aatccagaag tacaagcagt ctgttcaagg agcagggaca      540
agttacagga atgttctcca agcagcaatc caaaagagtc tcaaggatcc ttcaaataac      600
ttcaggctcc ataatgggag aagtaaagaa caaagactta aggaacagct tggcgctcaa      660
caagaaccag tgaagaaatc cattcaggaa tcagaagctt ttttgctca gacataacct      720
gaagagagat acaagatgaa gagcaagccc ctaggaatct gcctgataat cgattgcatt      780
ggcaatgaga cagaaaacgc ccactcctgg atcttcacat tgaactcaat ggctacatgt      840
atgattggaa cagcagagtt tctgccagg agaaatatta tgtttggtg cagcacactc      900
tgagaaagaa acttatectc tcctacacat aa                                  932

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&lt;210&gt; 8

&lt;211&gt; 300

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 8

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Met Ser Ala Glu Val Ile His Gln Val Glu Glu Ala Leu Asp Thr Asp
 1             5             10            15
Glu Lys Glu Met Leu Leu Phe Leu Cys Arg Asp Val Ala Ile Asp Val
          20             25            30
Val Pro Pro Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly
          35             40            45
Lys Leu Ser Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg
          50             55            60
Phe Asp Leu Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu

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65	70	75	80
Thr His Leu Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu			
	85	90	95
Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu			
	100	105	110
Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu			
	115	120	125
Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val			
	130	135	140
Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His			
145	150	155	160
Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln			
	165	170	175
Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys			
	180	185	190
Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Leu His Asn Gly Arg Ser			
	195	200	205
Lys Glu Gln Arg Leu Lys Glu Gln Leu Gly Ala Gln Gln Glu Pro Val			
	210	215	220
Lys Lys Ser Ile Gln Glu Ser Glu Ala Phe Leu Pro Gln Ser Ile Pro			
225	230	235	240
Glu Glu Arg Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile			
	245	250	255
Ile Asp Cys Ile Gly Asn Glu Thr Glu Asn Ala His Ser Trp Ile Phe			
	260	265	270
Thr Leu Asn Ser Met Ala Thr Cys Met Ile Gly Thr Ala Glu Phe Leu			
	275	280	285
Pro Arg Arg Asn Ile Met Phe Gly Cys Ser Thr Leu			
290	295	300	

&lt;210&gt; 9

&lt;211&gt; 2056

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 9

cgagtctcaa ctaaaaggga ctcccggagc taggggtggg gactcggcct cacacagtga	60
gtgccggcta ttggactttt gtccagtgc agctgagaca acaaggacca cgggaggagg	120

tgtaggagag aagcgccgcg aacagcgatc gcccagcacc aagtcgcgtt ccaggctttc	180
ggttttctttg cctccatctt ggggtgcgcct tcccggcgtc taggggagcg aaggtctgagg	240
tggcagcggc aggagagtcc ggccgcgaca ggacgaactc cccactgga aaggattctg	300
aaagaaatga agtcagccct cagaaatgaa gttgactgcc tgctggcttt ctgttgactg	360
gcccggagct gtactgcaag acccttgtga gcttccctag tctaagagta ggatgtctgc	420
tgaagtcac catcagggtt aagaagcact tgatacagat gagaaggaga tgctgctctt	480
tttgtgccgg gatgttgcta tagatgtggg tccacctaata gtcagggacc ttctggatat	540
tttacgggaa agaggttaagc tgtctgtcgg ggacttggct gaactgctct acagagttag	600
gcgatttgac ctgctcaaac gtatcttgaa gatggacaga aaagctgtgg agacccacct	660
gctcaggaac cctcaccttg ttctggacta tagagtgtct atggcagaga ttggtgagga	720
tttgataaaa tctgatgtgt cctcattaat ttctctcatg aaggattaca tgggccgagg	780
caagataagc aaggagaaga gtttcttgga ccttgtggtt gagttggaga aactaaatct	840
ggttgcccc gatcaactgg atttattaga aaaatgccta aagaacatcc acagaataga	900
cctgaagaca aaaatccaga agtacaagca gtctgttcaa ggagcagggg caagttacag	960
gaatgttctc caagcagcaa tccaaaagag tctcaaggat ccttcaaata acttcaggag	1020
catacctgaa gagagataca agatgaagag caagccccta ggaatctgcc tgataatcga	1080
ttgcattggc aatgagacag agcttcttcg agacacctc acttccctgg gctatgaagt	1140
ccagaaattc ttgcatctca gtatgcattg tatatcccag attcttggcc aatttgctg	1200
tatgcccag caccgagact acgacagctt tgtgtgtgtc ctggtgagcc gaggaggctc	1260
ccagagtgtg tatggtgtgg atcagactca ctgagggtc cccctgcac acatcaggag	1320
gatgttcatg ggagattcat gcccttatct agcaggggag ccaaagatgt tttttattca	1380
gaactatgtg gtgtcagagg gccagctgga ggacagcagc ctcttgagg tggatgggcc	1440
agcgatgaag aatgtggaat tcaaggctca gaagcgaggg ctgtgcacag ttcaccgaga	1500
agctgacttc ttctggagcc tgtgtactgc ggacatgtcc ctgctggagc agtctcacag	1560
ctcacgctcc ctgtacctgc agtgcccttc ccagaaactg agacaagaaa gaaaacgccc	1620
actcctggat ctccacattg aactcaatgg ctacatgtat gattggaaca gcagagtttc	1680
tgccaaggag aaatattatg tttggctgca gcacactctg agaaagaaac ttatcctctc	1740
ctacacataa gaaacaaaaa ggctgggctg agtggctcac acctgtaac ccagcacttt	1800
gggaggccaa ggagggcaga tcacttcagg tcaggagtcc gagaccagcc tggccaacat	1860
ggtaaacgct gtccctagta aaaatacaaa aattagctgg gtgtgggtgt gggtagctgt	1920
attcccagtt acttgggagg ctgaggtggg aggatctttt gaacccagga gttcagggtc	1980
atagcatgct gtgattgtgc ctacgaatag cactgcata ccaacctggg caatatagca	2040
agatcccatc tcttta	2056

&lt;210&gt; 10

&lt;211&gt; 445

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 10

Met Ser Ala Glu Val Ile His Gln Val Glu Glu Ala Leu Asp Thr Asp  
 1 5 10 15  
 Glu Lys Glu Met Leu Leu Phe Leu Cys Arg Asp Val Ala Ile Asp Val  
 20 25 30  
 Val Pro Pro Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly  
 35 40 45  
 Lys Leu Ser Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg  
 50 55 60  
 Phe Asp Leu Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu  
 65 70 75 80  
 Thr His Leu Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu  
 85 90 95  
 Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu  
 100 105 110  
 Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu  
 115 120 125  
 Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val  
 130 135 140  
 Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His  
 145 150 155 160  
 Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln  
 165 170 175  
 Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys  
 180 185 190  
 Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Ser Ile Pro Glu Glu Arg  
 195 200 205  
 Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile Ile Asp Cys  
 210 215 220  
 Ile Gly Asn Glu Thr Glu Leu Leu Arg Asp Thr Phe Thr Ser Leu Gly  
 225 230 235 240  
 Tyr Glu Val Gln Lys Phe Leu His Leu Ser Met His Gly Ile Ser Gln  
 245 250 255  
 Ile Leu Gly Gln Phe Ala Cys Met Pro Glu His Arg Asp Tyr Asp Ser  
 260 265 270  
 Phe Val Cys Val Leu Val Ser Arg Gly Gly Ser Gln Ser Val Tyr Gly  
 275 280 285  
 Val Asp Gln Thr His Ser Gly Leu Pro Leu His His Ile Arg Arg Met

290                      295                      300  
 Phe Met Gly Asp Ser Cys Pro Tyr Leu Ala Gly Lys Pro Lys Met Phe  
 305                      310                      315                      320  
 Phe Ile Gln Asn Tyr Val Val Ser Glu Gly Gln Leu Glu Asp Ser Ser  
                          325                      330                      335  
 Leu Leu Glu Val Asp Gly Pro Ala Met Lys Asn Val Glu Phe Lys Ala  
                          340                      345                      350  
 Gln Lys Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp Phe Phe Trp  
                          355                      360                      365  
 Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser His Ser Ser  
                          370                      375                      380  
 Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg Gln Glu Arg  
 385                      390                      395                      400  
 Lys Arg Pro Leu Leu Asp Leu His Ile Glu Leu Asn Gly Tyr Met Tyr  
                          405                      410                      415  
 Asp Trp Asn Ser Arg Val Ser Ala Lys Glu Lys Tyr Tyr Val Trp Leu  
                          420                      425                      430  
 Gln His Thr Leu Arg Lys Lys Leu Ile Leu Ser Tyr Thr  
                          435                      440                      445

&lt;210&gt; 11

&lt;211&gt; 1057

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 11

atgtctgctg aagtcaccca tcagggttgaa gaagcacttg atacagatga gaaggagatg 60  
 ctgctctttt tgtgccggga tgttgctata gatgtggttc cacctaattg cagggacctt 120  
 ctggatattt tacgggaaag aggtaagctg tctgtcgggg acttggtgta actgctctac 180  
 agagtgagga gatttgacct gctcaaactg atcttgaaga tggacagaaa agctgtggag 240  
 acccacctgc tcaggaaccc tcaccttggt tcggactata gagtgctgat ggcagagatt 300  
 ggtgaggatt tggataaatc tgatgtgtcc tcattaattt tcctcatgaa ggattacatg 360  
 ggccgaggca agataagcaa ggagaagagt ttcttgacc ttgtggttga gttggagaaa 420  
 ctaaactctgg ttgccccaga tcaactggat ttattagaaa aatgcctaaa gaacatccac 480  
 agaatagacc tgaagacaaa aatccagaag tacaagcagt ctgttcaagg agcagggaca 540  
 agttacagga atgtttctca agcagcaatc caaaagagtc tcaaggatcc ttcaaataac 600  
 ttcaggagca tacctgaaga gagatacaag atgaagagca agcccctagg aatctgcctg 660  
 ataatcgatt gcattggcaa tgagacagaa ctatgtggtg tcagagggcc agctggagga 720



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cagcagcctc ttggagggtgg atgggccagc gatgaagaat gtggaattca aggctcagaa      780
gcgagggtctg tgcacagttc accgagaagc tgacttcttc tggagcctgt gtactgcgga      840
catgtccctg ctggagcagt ctcacagctc accgtccctg tacctgcagt gcctctccca      900
gaaactgaga caagaaagaa aacgcccact cctggatctt cacattgaac tcaatggcta      960
catgtatgat tggaacagca gagtttctgc caaggagaaa tattatgttt ggctgcagca     1020
cactctgaga aagaaactta tcctctccta cacataa                                1057

```

&lt;210&gt; 12

&lt;211&gt; 270

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 12

```

Met Ser Ala Glu Val Ile His Gln Val Glu Glu Ala Leu Asp Thr Asp
  1              5              10              15
Glu Lys Glu Met Leu Leu Phe Leu Cys Arg Asp Val Ala Ile Asp Val
      20              25              30
Val Pro Pro Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly
      35              40              45
Lys Leu Ser Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg
      50              55              60
Phe Asp Leu Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu
      65              70              75              80
Thr His Leu Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu
      85              90              95
Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu
      100             105             110
Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu
      115             120             125
Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val
      130             135             140
Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His
      145             150             155             160
Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln
      165             170             175
Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys
      180             185             190
Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Ser Ile Pro Glu Glu Arg

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195	200	205
Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile Ile Asp Cys		
210	215	220
Ile Gly Asn Glu Thr Glu Leu Cys Gly Val Arg Gly Pro Ala Gly Gly		
225	230	235
Gln Gln Pro Leu Gly Gly Gly Trp Ala Ser Asp Glu Glu Cys Gly Ile		
245	250	255
Gln Gly Ser Glu Ala Arg Ala Val His Ser Ser Pro Arg Ser		
260	265	270

&lt;210&gt; 13

&lt;211&gt; 1458

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 13

tagtctaaga gtaggatgtc tgctgaagtc atccatcagg ttgaagaagc acttgataca	60
gatgagaagg agatgctgct ctttttgtgc cgggatgttg ctatagatgt ggttccacct	120
aatgtcaggg accttctgga tattttacgg gaaagaggta agctgtctgt cggggacttg	180
gctgaactgc tctacagagt gaggcgattt gacctgtca aacgtatctt gaagatggac	240
agaaaagctg tggagacca cctgctcagg aacctcacc ttgtttcgga ctatagagt	300
ctgatggcag agattggtga ggatttgat aaatctgatg tgcctcatt aattttctc	360
atgaaggatt acatgggccc aggcaagata agcaaggaga agagtttctt ggaccttg	420
gttgagttgg agaaactaaa tctggttgcc ccagatcaac tggatttatt agaaaaatgc	480
ctaaagaaca tccacagaat agacctgaag acaaaaatcc agaagtacaa gcagtctgtt	540
caaggagcag ggacaagtta caggaatgtt ctccaagcag caatccaaaa gagtctcaag	600
gacccctcaa ataacttcag gctccataat gggagaagta aagaacaaag acttaaggaa	660
cagcttggcg ctcaacaaga accagtgaag aaatccattc aggaatcaga agcttttttg	720
cctcagagca tacctgaaga gagatacaag atgaagagca agcccctagg aatctgcctg	780
ataatcgatt gcattggcaa tgagacagag cttcttcgag acaccttcac ttccctgggc	840
tatgaagtcc agaaattctt gcatctcagt atgcatggta tatcccagat tcttggccaa	900
tttgectgta tgcccagca ccgagactac gacagctttg tgtgtgtcct ggtgagccga	960
ggaggctccc agagtgtgta tgggtgtggat cagactcact cagggtctccc cctgcatcac	1020
atcaggagga tgttcatggg agattcatgc ccttatctag cagggaagcc aaagatgttt	1080
tttattcaga actatgtggt gtcagagggc cagctggagg acagcagcct cttggagggtg	1140
gatgggcccag cgatgaagaa tgtggaattc aaggctcaga agcaggggct gtgcacagtt	1200
caccgagaag ctgacttctt ctggagcctg tgtactgcgg acatgtccct gctggagcag	1260
tctcacagct caccgtccct gtacctgcag tgcctctccc agaaactgag acaagaaaga	1320

aaacgcccac tcttgatct tcacattgaa ctcaatggct acatgtatga ttggaacagc 1380  
 agagtttctg ccaaggagaa atattatgtc tggctgcagc acactctgag aaagaaactt 1440  
 atcctctcct acacataa 1458

<210> 14

<211> 480

<212> PRT

<213> Homo Sapiens

<400> 14

Met Ser Ala Glu Val Ile His Gln Val Glu Glu Ala Leu Asp Thr Asp  
 1 5 10 15  
 Glu Lys Glu Met Leu Leu Phe Leu Cys Arg Asp Val Ala Ile Asp Val  
 20 25 30  
 Val Pro Pro Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly  
 35 40 45  
 Lys Leu Ser Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg  
 50 55 60  
 Phe Asp Leu Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu  
 65 70 75 80  
 Thr His Leu Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu  
 85 90 95  
 Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu  
 100 105 110  
 Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu  
 115 120 125  
 Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val  
 130 135 140  
 Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His  
 145 150 155 160  
 Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln  
 165 170 175  
 Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys  
 180 185 190  
 Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Leu His Asn Gly Arg Ser  
 195 200 205  
 Lys Glu Gln Arg Leu Lys Glu Gln Leu Gly Ala Gln Gln Glu Pro Val  
 210 215 220

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<210> 15
<211> 1443
<212> DNA
<213> Homo Sapiens
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&lt;400&gt; 15

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ctggatattt tacgggaaaag aggttaagctg tctgtcgggg acttggtga actgctctac     180
agagtggaggc gatttgacct gctcaaactg atcttgaaga tggacagaaa agctgtggag      240
accacactgc tcaggaaccc tcacctgtt tcggactata gagtgtgat ggcagagatt      300
ggtgaggatt tggataaatc tgatgtgtcc tcattaattt tcctcatgaa ggattacatg      360
ggccgaggca agataagcaa ggagaagagt ttcttgacc ttgtggttga gttggagaaa      420
ctaaatctgg ttgccccaga tcaactggat ttattagaaa aatgcctaaa gaacatccac     480
agaatagacc tgaagacaaa aatccagaag tacaagcagt ctgttcaagg agcagggaca      540
agttacagga atgttctcca agcagcaatc caaaagagtc tcaaggatcc ttcaaataac     600
ttcaggctcc ataatgggag aagtaaagaa caaagactta aggaacagct tggcgctcaa      660
caagaaccag tgaagaaatc cattcaggaa tcagaagctt ttttgctca gagcatacct      720
gaagagagat acaagatgaa gagcaagccc ctaggaatct gcctgataat cgattgcatt      780
ggcaatgaga cagagcttct tcgagacacc ttcaattccc tgggctatga agtccagaaa      840
ttcttgcatc tcagtatgca tggatatatc cagattcttg gccaatattg ctgtatgcc      900
gagcaccgag actacgacag ctttgtgtgt gtccctgggtga gccgaggagg ctcccagagt     960
gtgtatggtg tggatcagac tcactcaggg ctccccctgc atcacatcag gaggatgttc    1020
atgggagatt catgccctta tctagcaggg aagccaaaga tgttttttat tcagaactat     1080
gtggtgtcag agggccagct ggaggacagc agcctcttgg aggtggatgg gccagcgatg     1140
aagaatgtgg aattcaaggc tcagaagcga gggtgtgca cagttcaccg agaagctgac      1200
ttcttctgga gcctgtgtac tgcggacatg tccctgctgg agcagtctca cagctcacca     1260
tcctgtacc tgcaagtgcct ctcccagaaa ctgagacaag aaagaaaacg cccactcctg     1320
gatcttcaca ttgaactcaa tggctacatg tatgattgga acagcagagt ttctgccaag     1380
gagaaatatt atgtctggct gcagcacact ctgagaaaga aacttatcct ctctacaca     1440
taa                                                                    1443

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&lt;210&gt; 16

&lt;211&gt; 480

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 16

```

Met Ser Ala Glu Val Ile His Gln Val Glu Glu Ala Leu Asp Thr Asp
  1             5             10             15
Glu Lys Glu Met Leu Leu Phe Leu Cys Arg Asp Val Ala Ile Asp Val
          20             25             30
Val Pro Pro Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly

```

35	40	45
Lys Leu Ser Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg		
50	55	60
Phe Asp Leu Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu		
65	70	75
Thr His Leu Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu		
85	90	95
Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu		
100	105	110
Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu		
115	120	125
Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val		
130	135	140
Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His		
145	150	155
Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln		
165	170	175
Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys		
180	185	190
Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Leu His Asn Gly Arg Ser		
195	200	205
Lys Glu Gln Arg Leu Lys Glu Gln Leu Gly Ala Gln Gln Glu Pro Val		
210	215	220
Lys Lys Ser Ile Gln Glu Ser Glu Ala Phe Leu Pro Gln Ser Ile Pro		
225	230	235
Glu Glu Arg Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile		
245	250	255
Ile Asp Cys Ile Gly Asn Glu Thr Glu Leu Leu Arg Asp Thr Phe Thr		
260	265	270
Ser Leu Gly Tyr Glu Val Gln Lys Phe Leu His Leu Ser Met His Gly		
275	280	285
Ile Ser Gln Ile Leu Gly Gln Phe Ala Cys Met Pro Glu His Arg Asp		
290	295	300
Tyr Asp Ser Phe Val Cys Val Leu Val Ser Arg Gly Gly Ser Gln Ser		
305	310	315
Val Tyr Gly Val Asp Gln Thr His Ser Gly Leu Pro Leu His His Ile		
325	330	335
Arg Arg Met Phe Met Gly Asp Ser Cys Pro Tyr Leu Ala Gly Lys Pro		

340 345 350  
 Lys Met Phe Phe Ile Gln Asn Tyr Val Val Ser Glu Gly Gln Leu Glu  
 355 360 365  
 Asp Ser Ser Leu Leu Glu Val Asp Gly Pro Ala Met Lys Asn Val Glu  
 370 375 380  
 Phe Lys Ala Gln Lys Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp  
 385 390 395 400  
 Phe Phe Trp Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser  
 405 410 415  
 His Ser Ser Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg  
 420 425 430  
 Gln Glu Arg Lys Arg Pro Leu Leu Asp Leu His Ile Glu Leu Asn Gly  
 435 440 445  
 Tyr Met Tyr Asp Trp Asn Ser Arg Val Ser Ala Lys Glu Lys Tyr Tyr  
 450 455 460  
 Val Trp Leu Gln His Thr Leu Arg Lys Lys Leu Ile Leu Ser Tyr Thr  
 465 470 475 480

&lt;210&gt; 17

&lt;211&gt; 2039

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 17

agtctcaact aaaagggact cccggagcta ggggtgggga ctccggcctca cacagtgagt 60  
 gccggctatt ggacttttgt ccagtgcacag ctgagacaac aaggaccacg ggaggagggtg 120  
 taggagagaa ggcgcgcgaa cagcgatcgc ccagcaccaa gtccgcttcc aggcctttcgg 180  
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 gcagcggcag gagagtccgg ccgcgacagg acgaactccc cactggaaa ggattctgaa 300  
 agaaatgaag tcagccctca gaaatgaagt tgactgcctg ctggctttct gttgactggc 360  
 ccggagctgt actgcaagac ccttgtgagc ttccctagtc taagagtagg atgtctgctg 420  
 aagtcattcca tcagggtgaa gaagcacttg atacagatga gaaggagatg ctgctctttt 480  
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 gatttgacct gctcaaacgt atcttgaaga tggacagaaa agctgtggag acccacctgc 660  
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 tggataaatc tgatgtgtcc tcattaattt tcctcatgaa ggattacatg ggccgaggca 780  
 agataagcaa ggagaagagt ttcttgacc ttgtgggtga gttggagaaa ctaaatctgg 840

```

ttgccccaga tcaactggat ttattagaaa aatgcctaaa gaacatccac agaatagacc      900
tgaagacaaa aatccagaag tacaagcagt ctgttcaagg agcagggaca agttacagga      960
atgttctcca agcagcaatc caaaagagtc tcaaggatcc ttcaaataac ttcaggctcc     1020
ataatgggag aagtaaagaa caaagactta aggaacagct tggcgctcaa caagaaccag     1080
tgaagaaatc cattcaggaa tcagaagctt ttttgcctca gagcatacct gaagagagat     1140
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cagagcttct tcgagacacc ttcacttccc tgggctatga agtccagaaa ttcttgcac     1260
tcagtatgca tggatatatcc cagattcttg gccaatattgc ctgtatgccc gagcaccgag     1320
actacgacag ctttgtgtgt gtccctgggtga gccgaggagg ctcccagagt gtgtatgggtg     1380
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agggccagct ggaggacagc agcctcttgg aggtggatgg gccagcgatg aagaatgtgg     1560
aattcaaggc tcagaagcga gggctgtgca cagttcaccg agaagctgac ttcttctgga     1620
gcctgtgtac tgccgacatg tccctgctgg agcagtctca cagctcaccg tcctgttacc     1680
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ttgaactcaa tggctacatg tatgattgga acagcagagt ttctgccaag gagaaatatt     1800
atgtctggct gcagcacact ctgagaaaga aacttatcct ctctacaca taagaaacca     1860
aaaggctggg cgtagtggct cacacctgta atcccagcac tttgggaggg caaggagggc     1920
agatcacttc aggtcaggag ttcgagacca gcctggccaa catggtaaac gctgtcccta     1980
gtaaaaatac aaaaattaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa     2039

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&lt;210&gt; 18

&lt;211&gt; 480

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 18

```

Met Ser Ala Glu Val Ile His Gln Val Glu Glu Ala Leu Asp Thr Asp
 1             5             10            15
Glu Lys Glu Met Leu Leu Phe Leu Cys Arg Asp Val Ala Ile Asp Val
      20             25            30
Val Pro Pro Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly
      35             40            45
Lys Leu Ser Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg
      50             55            60
Phe Asp Leu Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu
65             70             75            80
Thr His Leu Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu

```



	85		90		95										
Met	Ala	Glu	Ile	Gly	Glu	Asp	Leu	Asp	Lys	Ser	Asp	Val	Ser	Ser	Leu
	100						105						110		
Ile	Phe	Leu	Met	Lys	Asp	Tyr	Met	Gly	Arg	Gly	Lys	Ile	Ser	Lys	Glu
	115						120						125		
Lys	Ser	Phe	Leu	Asp	Leu	Val	Val	Glu	Leu	Glu	Lys	Leu	Asn	Leu	Val
	130						135						140		
Ala	Pro	Asp	Gln	Leu	Asp	Leu	Leu	Glu	Lys	Cys	Leu	Lys	Asn	Ile	His
145						150					155			160	
Arg	Ile	Asp	Leu	Lys	Thr	Lys	Ile	Gln	Lys	Tyr	Lys	Gln	Ser	Val	Gln
						165					170			175	
Gly	Ala	Gly	Thr	Ser	Tyr	Arg	Asn	Val	Leu	Gln	Ala	Ala	Ile	Gln	Lys
						180					185			190	
Ser	Leu	Lys	Asp	Pro	Ser	Asn	Asn	Phe	Arg	Leu	His	Asn	Gly	Arg	Ser
	195						200						205		
Lys	Glu	Gln	Arg	Leu	Lys	Glu	Gln	Leu	Gly	Ala	Gln	Gln	Glu	Pro	Val
	210						215						220		
Lys	Lys	Ser	Ile	Gln	Glu	Ser	Glu	Ala	Phe	Leu	Pro	Gln	Ser	Ile	Pro
225						230					235			240	
Glu	Glu	Arg	Tyr	Lys	Met	Lys	Ser	Lys	Pro	Leu	Gly	Ile	Cys	Leu	Ile
						245					250			255	
Ile	Asp	Cys	Ile	Gly	Asn	Glu	Thr	Glu	Leu	Leu	Arg	Asp	Thr	Phe	Thr
						260					265			270	
Ser	Leu	Gly	Tyr	Glu	Val	Gln	Lys	Phe	Leu	His	Leu	Ser	Met	His	Gly
	275						280						285		
Ile	Ser	Gln	Ile	Leu	Gly	Gln	Phe	Ala	Cys	Met	Pro	Glu	His	Arg	Asp
	290						295						300		
Tyr	Asp	Ser	Phe	Val	Cys	Val	Leu	Val	Ser	Arg	Gly	Gly	Ser	Gln	Ser
305						310					315			320	
Val	Tyr	Gly	Val	Asp	Gln	Thr	His	Ser	Gly	Leu	Pro	Leu	His	His	Ile
						325					330			335	
Arg	Arg	Met	Phe	Met	Gly	Asp	Ser	Cys	Pro	Tyr	Leu	Ala	Gly	Lys	Pro
						340					345			350	
Lys	Met	Phe	Phe	Ile	Gln	Asn	Tyr	Val	Val	Ser	Glu	Gly	Gln	Leu	Glu
						355							360		365
Asp	Ser	Ser	Leu	Leu	Glu	Val	Asp	Gly	Pro	Ala	Met	Lys	Asn	Val	Glu
	370						375						380		
Phe	Lys	Ala	Gln	Lys	Arg	Gly	Leu	Cys	Thr	Val	His	Arg	Glu	Ala	Asp

385	390	395	400
Phe Phe Trp Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser			
	405	410	415
His Ser Ser Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg			
	420	425	430
Gln Glu Arg Lys Arg Pro Leu Leu Asp Leu His Ile Glu Leu Asn Gly			
	435	440	445
Tyr Met Tyr Asp Trp Asn Ser Arg Val Ser Ala Lys Glu Lys Tyr Tyr			
	450	455	460
Val Trp Leu Gln His Thr Leu Arg Lys Lys Leu Ile Leu Ser Tyr Thr			
465	470	475	480

&lt;210&gt; 19

&lt;211&gt; 1443

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 19

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ctgctctttt tgtgccggga tgttgctata gatgtggttc cacctaattg cagggacctt	120
ctggatattt tacgggaaag aggtaagctg tctgtcgggg acttggtgga actgctctac	180
agagtgaggg gatttgacct gctcaaactg atcttgaaga tggacagaaa agctgtggag	240
accacactgc tcagggaacc tcacctgtt tcggactata gagtgtgat ggcagagatt	300
ggtgaggatt tggataaatc tgatgtgtcc tcattaattt tcctcatgaa ggattacatg	360
ggccgaggca agataagcaa ggagaagagt ttcttgacc ttgtggttga gttggagaaa	420
ctaaatctgg ttgccccaga tcaactggat ttattagaaa aatgcctaaa gaacatccac	480
agaatagacc tgaagacaaa aatccagaag tacaagcagt ctgttcaagg agcagggaca	540
agttacagga atgttctcca agcagcaatc caaaagagtc tcaaggatcc ttcaaataac	600
ttcaggctcc ataatgggag aagtaaagaa caaagactta aggaacagct tggcgctcaa	660
caagaaccag tgaagaaatc cattcaggaa tcagaagctt ttttgcctca gagcatacct	720
gaagagagat acaagatgaa gagcaagccc ctaggaatct gcctgataat cgattgcatt	780
ggcaatgaga cagagcttct tcgagacacc ttcaactccc tgggctatga agtccagaaa	840
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gagcaccgag actacgacag ctttgtgtgt gtcctgggtga gccgaggagg ctcccagagt	960
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gtggtgtcag agggccagct ggaggacagc agcctcttgg aggtggatgg gccagcgatg	1140
aagaatgtgg aattcaaggc tcagaagcga gggctgtgca cagttcaccg agaagctgac	1200

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gatcttcaca ttgaactcaa tggctacatg tatgattgga acagcagagt ttctgccaag      1380
gagaaatatt atgtttggct gcagcacact ctgagaaaga aacttatacct ctctacaca      1440
taa                                                                    1443

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&lt;210&gt; 20

&lt;211&gt; 480

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 20

```

Met Ser Ala Glu Val Ile His Gln Val Glu Glu Ala Leu Asp Thr Asp
 1              5              10              15
Glu Lys Glu Met Leu Leu Phe Leu Cys Arg Asp Val Ala Ile Asp Val
      20              25              30
Val Pro Pro Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly
      35              40              45
Lys Leu Ser Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg
      50              55              60
Phe Asp Leu Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu
65              70              75              80
Thr His Leu Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu
      85              90              95
Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu
      100             105             110
Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu
      115             120             125
Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val
      130             135             140
Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His
145             150             155             160
Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln
      165             170             175
Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys
      180             185             190
Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Leu His Asn Gly Arg Ser
      195             200             205

```

Lys Glu Gln Arg Leu Lys Glu Gln Leu Gly Ala Gln Gln Glu Pro Val  
 210 215 220  
 Lys Lys Ser Ile Gln Glu Ser Glu Ala Phe Leu Pro Gln Ser Ile Pro  
 225 230 235 240  
 Glu Glu Arg Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile  
 245 250 255  
 Ile Asp Cys Ile Gly Asn Glu Thr Glu Leu Leu Arg Asp Thr Phe Thr  
 260 265 270  
 Ser Leu Gly Tyr Glu Val Gln Lys Phe Leu His Leu Ser Met His Gly  
 275 280 285  
 Ile Ser Gln Ile Leu Gly Gln Phe Ala Cys Met Pro Glu His Arg Asp  
 290 295 300  
 Tyr Asp Ser Phe Val Cys Val Leu Val Ser Arg Gly Gly Ser Gln Ser  
 305 310 315 320  
 Val Tyr Gly Val Asp Gln Thr His Ser Gly Leu Pro Leu His His Ile  
 325 330 335  
 Arg Arg Met Phe Met Gly Asp Ser Cys Pro Tyr Leu Ala Gly Lys Pro  
 340 345 350  
 Lys Met Phe Phe Ile Gln Asn Tyr Val Val Ser Glu Gly Gln Leu Glu  
 355 360 365  
 Asp Ser Ser Leu Leu Glu Val Asp Gly Pro Ala Met Lys Asn Val Glu  
 370 375 380  
 Phe Lys Ala Gln Lys Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp  
 385 390 395 400  
 Phe Phe Trp Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser  
 405 410 415  
 His Ser Ser Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg  
 420 425 430  
 Gln Glu Arg Lys Arg Pro Leu Leu Asp Leu His Ile Glu Leu Asn Gly  
 435 440 445  
 Tyr Met Tyr Asp Trp Asn Ser Arg Val Ser Ala Lys Glu Lys Tyr Tyr  
 450 455 460  
 Val Trp Leu Gln His Thr Leu Arg Lys Lys Leu Ile Leu Ser Tyr Thr  
 465 470 475 480

&lt;210&gt; 21

&lt;211&gt; 1350

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 21

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atgtctgctg aagtcaccca tcagggtgaa gaagcacttg atacagatga gaaggagatg      60
ctgctctttt tgtgccggga tgttgctata gatgtggttc cacctaattg cagggacctt      120
ctggatatatt tacgggaaag aggtaagctg tctgtcgggg acttggtga actgctctac      180
agagtgaggc gatttgacct gctcaaactg atcttgaaga tggacagaaa agctgtggag      240
accacactgc tcaggaacct tcacctgtt tcggactata gagtgtgat ggcagagatt      300
ggtgaggatt tggataaatc tgatgtgtcc tcattaattt tcctcatgaa ggattacatg      360
ggccgaggca agataagcaa ggagaagagt ttcttgacc ttgtggttga gttggagaaa      420
ctaaatctgg ttgccccaga tcaactggat ttattagaaa aatgcctaaa gaacatccac      480
agaatagacc tgaagacaaa aatccagaag tacaagcagt ctgttcaagg agcagggaca      540
agttacagga atgttctcca agcagcaatc caaaagagtc tcaaggatcc ttcaaataac      600
ttcaggctcc ataatgggag aagtaaagaa caaagactta aggaacagct tggcgctcaa      660
caagaaccag tgaagaaatc cattcaggaa tcagaagctt ttttgctca gacataacct      720
gaagagagat acaagatgaa gagcaagccc ctaggaatct gcctgataat cgattgcatt      780
ggcaatgaga cagagcttct tcgagacacc ttcaactccc tgggctatga agtccagaaa      840
ttcttgcatc tcagtatgca tggatatcc cagattcttg gccaatctgc ctgtatgcc      900
gagcaccgag actacgacag ctttgtgtgt gtcttggtga gccgaggagg ctcccagagt      960
gtgtatggtg tggatcagac tcactcaggg ctccccctgc atcacatcag gaggatgttc     1020
atgggagatt catgccctta tctagcaggg aagccaaaga tgttttttat tcagaactat     1080
gtggtgtcag agggccagct ggaggacagc agcctcttgg aggtggatgg gccagcgatg     1140
aagaatgtgg aattcaaggc tcagaagcga gggctgtgca cagttcaccc agaagctgac     1200
ttcttctgga gcctgtgtac tgccgacatg tcctgtctgg agcagctctca cagctcaccc     1260
tcctgtacc tgcaagtgcct ctcccagaaa ctgagacaag aaagaaaacg cccactcctg     1320
gatcttcaca ttgaactcaa cggcctttag                                     1350

```

&lt;210&gt; 22

&lt;211&gt; 449

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 22

```

Met Ser Ala Glu Val Ile His Gln Val Glu Glu Ala Leu Asp Thr Asp
  1             5             10             15
Glu Lys Glu Met Leu Leu Phe Leu Cys Arg Asp Val Ala Ile Asp Val
          20             25             30
Val Pro Pro Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly

```

35	40	45
Lys Leu Ser Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg		
50	55	60
Phe Asp Leu Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu		
65	70	75
Thr His Leu Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu		
85	90	95
Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu		
100	105	110
Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu		
115	120	125
Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val		
130	135	140
Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His		
145	150	155
Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln		
165	170	175
Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys		
180	185	190
Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Leu His Asn Gly Arg Ser		
195	200	205
Lys Glu Gln Arg Leu Lys Glu Gln Leu Gly Ala Gln Gln Glu Pro Val		
210	215	220
Lys Lys Ser Ile Gln Glu Ser Glu Ala Phe Leu Pro Gln Ser Ile Pro		
225	230	235
Glu Glu Arg Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile		
245	250	255
Ile Asp Cys Ile Gly Asn Glu Thr Glu Leu Leu Arg Asp Thr Phe Thr		
260	265	270
Ser Leu Gly Tyr Glu Val Gln Lys Phe Leu His Leu Ser Met His Gly		
275	280	285
Ile Ser Gln Ile Leu Gly Gln Phe Ala Cys Met Pro Glu His Arg Asp		
290	295	300
Tyr Asp Ser Phe Val Cys Val Leu Val Ser Arg Gly Gly Ser Gln Ser		
305	310	315
Val Tyr Gly Val Asp Gln Thr His Ser Gly Leu Pro Leu His His Ile		
325	330	335
Arg Arg Met Phe Met Gly Asp Ser Cys Pro Tyr Leu Ala Gly Lys Pro		

340                      345                      350  
 Lys Met Phe Phe Ile Gln Asn Tyr Val Val Ser Glu Gly Gln Leu Glu  
 355                      360                      365  
 Asp Ser Ser Leu Leu Glu Val Asp Gly Pro Ala Met Lys Asn Val Glu  
 370                      375                      380  
 Phe Lys Ala Gln Lys Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp  
 385                      390                      395                      400  
 Phe Phe Trp Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser  
 405                      410                      415  
 His Ser Ser Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg  
 420                      425                      430  
 Gln Glu Arg Lys Arg Pro Leu Leu Asp Leu His Ile Glu Leu Asn Gly  
 435                      440                      445  
 Leu

&lt;210&gt; 23

&lt;211&gt; 1361

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 23

gcctctagat gcatgctcga gcggccgccg gtgtgctcta aagtagatgt gggtccacct 60  
 aatgtcaggg accttctgga tattttacgg gaaagaggta agctgtctgt cggggacttg 120  
 gctgaactgc tctacagagt gagggcattt gacctgctca aacgtatctt gaagatggac 180  
 agaaaagctg tggagaccca cctgctcagg aaccctcacc ttgtttcgga ctatagagt 240  
 ctgatggcag agattgggtga ggatttggat aaatctgatg tgtcctcatt aattttcctc 300  
 atgaaggatt acatgggccc aggcaagata agcaaggaga agagtttctt ggaccttggtg 360  
 gttgagttgg agaaactaaa tctgggtgcc ccagatcaac tggatttatt agaaaaatgc 420  
 ctaaagaaca tccacagaat agacctgaag acaaaaatcc agaagtacaa gcagtctgtt 480  
 caaggagcag ggacaagtta caggaatgtt ctccaagcag caatccaaaa gagtctcaag 540  
 gatccttcaa ataacttcag gctccataat gggagaagta aagaacaaag acttaaggaa 600  
 cagcttggcg ctcaacaaga accagtgaag aaatccattc aggaatcaga agcttttttg 660  
 cctcagagca tacctgaaga gagatacaag atgaagagca agcccctagg aatctgcctg 720  
 ataatcgatt gcattggcaa tgagacagag cttcttcgag acaccttcac ttccttgggc 780  
 tatgaagtcc agaaattctt gcatctcagt atgcatggta tatcccagat tcttggccaa 840  
 tttgctgtga tgcccagca cccagactac gacagctttg tgtgtgtcct ggtgagccga 900  
 ggaggctccc agagtgtgta tgggtgtggat cagactcact cagggtctccc cctgcatcac 960

```

atcaggagga tgttcattggg agattcatgc ccttatctag caggggaagcc aaagatgttt 1020
tttattcaga actatgtggt gtcagagggc cagctggagg acagcagctt cttggagggtg 1080
gatggggccag cgatgaagaa tgtggaattc aaggctcaga agcgagggct gtgcacagtt 1140
caccgagaag ctgacttctt ctggagcctg tgtactgagg acatgtccct gctggagcag 1200
tctcacagct caccgtccct gtacctgcag tgcctctccc agaaactgag acaagaaaga 1260
aaacgcccac tcctggatct tcacattgaa ctcaatggct acatgtatga ttcttttagag 1320
cacactggcg gccgttacta gtggatccga gctcggtagc a 1361

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&lt;210&gt; 24

&lt;211&gt; 442

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 24

```

Met Leu Glu Arg Pro Pro Val Cys Ser Lys Val Asp Val Val Pro Pro
  1              5              10              15
Asn Val Arg Asp Leu Leu Asp Ile Leu Arg Glu Arg Gly Lys Leu Ser
      20              25              30
Val Gly Asp Leu Ala Glu Leu Leu Tyr Arg Val Arg Arg Phe Asp Leu
      35              40              45
Leu Lys Arg Ile Leu Lys Met Asp Arg Lys Ala Val Glu Thr His Leu
      50              55              60
Leu Arg Asn Pro His Leu Val Ser Asp Tyr Arg Val Leu Met Ala Glu
      65              70              75              80
Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu Ile Phe Leu
      85              90              95
Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu Lys Ser Phe
      100              105              110
Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val Ala Pro Asp
      115              120              125
Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His Arg Ile Asp
      130              135              140
Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln Gly Ala Gly
      145              150              155              160
Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys Ser Leu Lys
      165              170              175
Asp Pro Ser Asn Asn Phe Arg Leu His Asn Gly Arg Ser Lys Glu Gln
      180              185              190

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Arg Leu Lys Glu Gln Leu Gly Ala Gln Gln Glu Pro Val Lys Lys Ser  
 195 200 205  
 Ile Gln Glu Ser Glu Ala Phe Leu Pro Gln Ser Ile Pro Glu Glu Arg  
 210 215 220  
 Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile Ile Asp Cys  
 225 230 235 240  
 Ile Gly Asn Glu Thr Glu Leu Leu Arg Asp Thr Phe Thr Ser Leu Gly  
 245 250 255  
 Tyr Glu Val Gln Lys Phe Leu His Leu Ser Met His Gly Ile Ser Gln  
 260 265 270  
 Ile Leu Gly Gln Phe Ala Cys Met Pro Glu His Arg Asp Tyr Asp Ser  
 275 280 285  
 Phe Val Cys Val Leu Val Ser Arg Gly Gly Ser Gln Ser Val Tyr Gly  
 290 295 300  
 Val Asp Gln Thr His Ser Gly Leu Pro Leu His His Ile Arg Arg Met  
 305 310 315 320  
 Phe Met Gly Asp Ser Cys Pro Tyr Leu Ala Gly Lys Pro Lys Met Phe  
 325 330 335  
 Phe Ile Gln Asn Tyr Val Val Ser Glu Gly Gln Leu Glu Asp Ser Ser  
 340 345 350  
 Phe Leu Glu Val Asp Gly Pro Ala Met Lys Asn Val Glu Phe Lys Ala  
 355 360 365  
 Gln Lys Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp Phe Phe Trp  
 370 375 380  
 Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser His Ser Ser  
 385 390 395 400  
 Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg Gln Glu Arg  
 405 410 415  
 Lys Arg Pro Leu Leu Asp Leu His Ile Glu Leu Asn Gly Tyr Met Tyr  
 420 425 430  
 Asp Ser Leu Glu His Thr Gly Gly Arg Tyr  
 435 440

&lt;210&gt; 25

&lt;211&gt; 1047

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 25

```

atggcagaga ttggtgagga tttggataaa tctgatgtgt cctcattaat tttcctcatg      60
aaggattaca tgggccgagg caagataagc aaggagaaga gtttcttgga ccttgtggtt      120
gagttggaga aactaaatct ggttgcccca gatcaactgg atttattaga aaaatgccta      180
aagaacatcc acagaataga cctgaagaca aaaatccaga agtacaagca gtctgttcaa      240
ggagcaggga caagttacag gaatgttctc caagcagcaa tccaaaagag tctcaaggat      300
ccttcaaata acttcaggga agaaccagtg aagaaatcca ttcaggaatc agaagctttt      360
ttgcctcaga gcatacctga agagagatac aagatgaaga gcaagcccct aggaatctgc      420
ctgataatcg attgcattgg caatgagaca gagcttcttc gagacacctt cacttccctg      480
ggctatgaag tccagaaatt cttgcatctc agtatgcatg gtatatccca gattcttggc      540
caatttgcct gtatgcccg gacccgagac tacgacagct ttgtgtgtgt cctggtgagc      600
cgaggagggt cccagagtgt gtatggtgtg gatcagactc actcagggtc cccctgcat      660
cacatcagga ggatgttcat gggagattca tgcccttata tagcaggga gccaagatg      720
ttttttatc agaactatgt ggtgtcagac ggccagctgg aggacagcag cctcttgag      780
gtggatgggc cagcgatgaa gaatgtggaa ttcaaggctc agaagcgagg gctgtgcaca      840
gttcaccgag aagctgactt cttctggagc ctgtgtactg cggacatgtc cctgctggag      900
cagtctcaca gctcaccgtc cctgtacctg cagtgcctct cccagaaact gagacaagaa      960
agggggacaa tccccggaag tggaattaca gagtcaaagg acatgcattt ttcaagcctc     1020
ggatgcatct tactagatgt cctatag                                           1047

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&lt;210&gt; 26

&lt;211&gt; 348

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 26

```

Met Ala Glu Ile Gly Glu Asp Leu Asp Lys Ser Asp Val Ser Ser Leu
  1              5              10              15
Ile Phe Leu Met Lys Asp Tyr Met Gly Arg Gly Lys Ile Ser Lys Glu
      20              25              30
Lys Ser Phe Leu Asp Leu Val Val Glu Leu Glu Lys Leu Asn Leu Val
      35              40              45
Ala Pro Asp Gln Leu Asp Leu Leu Glu Lys Cys Leu Lys Asn Ile His
      50              55              60
Arg Ile Asp Leu Lys Thr Lys Ile Gln Lys Tyr Lys Gln Ser Val Gln
      65              70              75              80
Gly Ala Gly Thr Ser Tyr Arg Asn Val Leu Gln Ala Ala Ile Gln Lys
      85              90              95

```

Ser Leu Lys Asp Pro Ser Asn Asn Phe Arg Glu Glu Pro Val Lys Lys  
 100 105 110  
 Ser Ile Gln Glu Ser Glu Ala Phe Leu Pro Gln Ser Ile Pro Glu Glu  
 115 120 125  
 Arg Tyr Lys Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile Ile Asp  
 130 135 140  
 Cys Ile Gly Asn Glu Thr Glu Leu Leu Arg Asp Thr Phe Thr Ser Leu  
 145 150 155 160  
 Gly Tyr Glu Val Gln Lys Phe Leu His Leu Ser Met His Gly Ile Ser  
 165 170 175  
 Gln Ile Leu Gly Gln Phe Ala Cys Met Pro Glu His Arg Asp Tyr Asp  
 180 185 190  
 Ser Phe Val Cys Val Leu Val Ser Arg Gly Gly Ser Gln Ser Val Tyr  
 195 200 205  
 Gly Val Asp Gln Thr His Ser Gly Leu Pro Leu His His Ile Arg Arg  
 210 215 220  
 Met Phe Met Gly Asp Ser Cys Pro Tyr Leu Ala Gly Lys Pro Lys Met  
 225 230 235 240  
 Phe Phe Ile Gln Asn Tyr Val Val Ser Asp Gly Gln Leu Glu Asp Ser  
 245 250 255  
 Ser Leu Leu Glu Val Asp Gly Pro Ala Met Lys Asn Val Glu Phe Lys  
 260 265 270  
 Ala Gln Lys Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp Phe Phe  
 275 280 285  
 Trp Ser Leu Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser His Ser  
 290 295 300  
 Ser Pro Ser Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg Gln Glu  
 305 310 315 320  
 Arg Gly Thr Ile Pro Gly Ser Gly Ile Thr Glu Ser Lys Asp Met His  
 325 330 335  
 Phe Ser Ser Leu Gly Cys Ile Leu Leu Asp Val Leu  
 340 345

&lt;210&gt; 27

&lt;211&gt; 1002

&lt;212&gt; DNA

&lt;213&gt; Homo Sapiens

&lt;400&gt; 27

```

accttggact gaaccactat tgaagattta tttgataata atttgtaagt ttttaactcag      60
tacctccctt ctgcttttat agttcaagga gcagggacaa gttacaggaa tgttctccaa      120
gcagcaatcc aaaagagtct caaggatcct tcaaataact tcaggctcca taatgggaga      180
agtaaagaac aaagacttaa ggaacagctt ggcgctcaac aagaaccagt gaagaaatcc      240
attcaggaat cagaagcttt tttgcctcag agcatacctg aagagagata caagatgaag      300
agcaagcccc taggaatctg cctgataatc gattgcattg gcaatgagac agagcttctt      360
cgagacacct tcacttcctt gggctatgaa gtccagaaat tcttgcatct cagtatgcat      420
ggatatatccc agattcttgg ccaatttgcc tgtatgcccg agcaccgaga ctacgacagc      480
tttgtgtgtg tcctggtgag ccgaggaggc tcccagagtg tgtatggtgt ggatcagact      540
cactcagggc tccccctgca tcacatcagg aggatgttca tgggagattc atgcccttat      600
ctagcagggg agccaaagat gttttttatt cagaactatg tgggtgtcaga gggccagctg      660
gaggacagca gcctcttgga ggtggatggg ccagcgatga agaattgtgga attcaaggct      720
cagaagcgag ggctgtgcac agttcaccga gaagctgact tcttctggag cctgtgtact      780
gcggacatgt ccctgctgga gcagtctcac agctcaccat ccctgtacct gcagtgcctc      840
tcccagaaac tgagacaaga aagaaaacgc ccactcctgg atcttcacat tgaactcaat      900
ggctacatgt atgattgaa cagcagagtt tctgccagg agaaatatta tgtctggctg      960
cagcacactc tgagaaagaa acttatcctc tctacacat aa                               1002

```

&lt;210&gt; 28

&lt;211&gt; 235

&lt;212&gt; PRT

&lt;213&gt; Homo Sapiens

&lt;400&gt; 28

```

Met Lys Ser Lys Pro Leu Gly Ile Cys Leu Ile Ile Asp Cys Ile Gly
 1              5              10              15
Asn Glu Thr Glu Leu Leu Arg Asp Thr Phe Thr Ser Leu Gly Tyr Glu
      20              25              30
Val Gln Lys Phe Leu His Leu Ser Met His Gly Ile Ser Gln Ile Leu
      35              40              45
Gly Gln Phe Ala Cys Met Pro Glu His Arg Asp Tyr Asp Ser Phe Val
      50              55              60
Cys Val Leu Val Ser Arg Gly Gly Ser Gln Ser Val Tyr Gly Val Asp
      65              70              75              80
Gln Thr His Ser Gly Leu Pro Leu His His Ile Arg Arg Met Phe Met
      85              90              95
Gly Asp Ser Cys Pro Tyr Leu Ala Gly Lys Pro Lys Met Phe Phe Ile

```

100	105	110
Gln Asn Tyr Val Val Ser Glu Gly Gln Leu Glu Asp Ser Ser Leu Leu		
115	120	125
Glu Val Asp Gly Pro Ala Met Lys Asn Val Glu Phe Lys Ala Gln Lys		
130	135	140
Arg Gly Leu Cys Thr Val His Arg Glu Ala Asp Phe Phe Trp Ser Leu		
145	150	155
Cys Thr Ala Asp Met Ser Leu Leu Glu Gln Ser His Ser Ser Pro Ser		
165	170	175
Leu Tyr Leu Gln Cys Leu Ser Gln Lys Leu Arg Gln Glu Arg Lys Arg		
180	185	190
Pro Leu Leu Asp Leu His Ile Glu Leu Asn Gly Tyr Met Tyr Asp Trp		
195	200	205
Asn Ser Arg Val Ser Ala Lys Glu Lys Tyr Tyr Val Trp Leu Gln His		
210	215	220
Thr Leu Arg Lys Lys Leu Ile Leu Ser Tyr Thr		
225	230	235

## INTERNATIONAL SEARCH REPORT

 International application No.  
PCT/US99/03558

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(6) : C12N 15/00, 5/00, 5/02; A01N 43/04; C07K 1/00; C07H 21/02 US CL : 435/320.1, 325, 455; 514/44; 530/350; 536/23.1 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/320.1, 325, 455; 514/44; 530/350; 536/23.1 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, CASPLUS, MEDLINE, WEST		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THOME, M. et al. Viral FLICE-inhibitory proteins (FLIPS) prevent apoptosis induced by death receptors. Nature. 03 April 1997, Vol. 386, pages 517-521, see entire document.	1-4, 6, 7, 9, 10-13, 24-30
Y,P	SATA, M. et al. Endothelial Cell Apoptosis Induced by Oxidized LDL is associated with the down regulation of the cellular caspase inhibitor FLIP. Journal of Biological Chemistry. 11 December 1998, Vol. 273, No. 50, pages 33103-33106, see entire document.	1-4, 6, 7, 9, 10-13, 24-30
X - Y	IRMLER, M. et al. Inhibition of Death Receptor Signals by Cellular FLIP. Nature. 10 July 1997, Vol. 388, pages 190-195, see entire document.	14-23 ----- 1-13, 24-30
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *B* earlier document published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *A* document member of the same patent family		
Date of the actual completion of the international search 07 MAY 1999		Date of mailing of the international search report 29 June 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer RAM R. SHUKLA Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/03558

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	HU, S. et al. I-FLICE, a Novel Inhibitor of Tumor Necrosis Factor Receptor-1 and CD-95-induced Apoptosis. Journal of Biological Chemistry. 11 July 1997, Vol. 272, No. 28, pages 17255-17257, see entire document.	14-23 ----- 1-13, 24-30
Y	ITOH, N. et al. A Novel Protein Domain Required for Apoptosis. Journal of Biological Chemistry. 23 May 1993. Vol. 268, No. 15, pages 10932-10937, see entire document.	1-4, 6, 7, 24-29
Y	NONOMURA, N. et al. FAS/APO-1 Mediated Apoptosis of Human Renal Cell Carcinoma. Biochemical Biophysical Research. Communication. 1996, Vol. 229, pages 945-951, see entire document.	1-4, 6, 7, 10-13, 24-29
X - Y	GOLTSEV, Y.V. et al. CASH, a novel caspase homologue with death effector domain. Journal of Biological Chemistry. 08 August 1997, Vol 272, No. 32, pages 19641-19644, see entire document.	14-23 ----- 1-10, 24-30

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/03558

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/03558

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions of groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, Claims 1-5, 9, 19-22, drawn to nucleic acids, vector, host cells and method of gene therapy.

Group II, Claims 1, 6-9, 18, 23, drawn to method of protein therapy.

Group III, Claims 10-13, drawn to method of screening for inhibitors.

Group IV, Claims 24-29, drawn to method of screening for inhibitors.

Group V, Claim 30, drawn to a method of inhibiting apoptosis in vitro.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature of the invention of group I, directed to a nucleic acid, a vector, a host cell, and a method of gene therapy, is different from the technical feature of invention of group II that is directed to a method of therapy using a protein. The process of these two groups are different from that of the group III because the invention of group III is drawn to a method of therapy of a transplanted organ in a recipient. The technical feature of the invention of group IV, drawn to an in vitro method of screening for inhibitors, is different from that of the group V that is drawn to an in vitro method of inhibiting apoptosis in a cell.